

Methods and Compositions for Regulating Lymphocyte Activity

Priority Information

This application claims priority to U.S. Provisional Application 60/168,112, filed
 5 November 30, 1999.

Background of the Invention

The lymphoid system performs critical functions in animals that include preventing and combating infection, and surveillance and immune elimination of tumor cells. Loss of immune function leads to an immunocompromised status that can predispose the host to serious and life-threatening disease. Functional abnormalities may be present in any of the elements that participate in mediating an immune response, e.g., cellular or humoral elements such as granulocytes, lymphocytes, complement, antibody, or cytokines.

The immune system is a network of cells adapted to protect the organism against pathogens and cells that are not recognized as "self." Once the immune system is activated, it enlists the participation of a variety of cells and molecules to mount an effector function designed to eliminate the "non-self" entity within the body. Lymphocytes are cells of the immune system that are capable of specifically recognizing and selectively eliminating foreign entities. By contrast to other cells of the immune system, such as neutrophils which are considered non-specific in their reactions to invaders, the characteristics of lymphocytes confer specificity, diversity, memory and self/nonself recognition to the immune response.

There are two major populations of lymphocytes: B lymphocytes and T lymphocytes. B lymphocytes originate and mature within the bone marrow and are responsible for formation of antibody molecules. T lymphocytes also arise from the bone marrow but mature in the thymus. There are two major subpopulations of T-cells: T helper cells and T cytotoxic cells. The two types of T cells can be distinguished by the presence of one of two membrane glycoproteins, either CD4 or CD8. The T-helper cells (which express CD4) when activated by antigen-complexes (foreign molecules coupled to special proteins) respond by secreting various growth factors known collectively as cytokines. These cytokines are signals that activate other cells of the immune system, including the T-cytotoxic cells. The T-cytotoxic cells (which express CD8) when activated, proliferate and differentiate into cytotoxic T lymphocytes (CTL) which are able to monitor for and eliminate from the body pathogenic cells, foreign cells, virus-infected cells, and tumor cells.

The normal development, maturation and differentiation of T lymphocytes are regulated by various peptide factors

5 Summary of the Invention

The present invention provides methods and compositions for modulating the immune function of an animal by administering an agent which mimics, or alternatively inhibits, *hedgehog*-mediated signal transduction. Thus, the subject method can be used as an immunosuppressive, e.g., by administering a *hedgehog* agonist, or as an immunostimulant, e.g.,
10 by administering a *hedgehog* antagonist

Wherein the subject method is carried out using a *hedgehog* therapeutic, the *hedgehog* therapeutic preferably a polypeptide including a *hedgehog* portion comprising at least a bioactive extracellular portion of a *hedgehog* protein, e.g., the *hedgehog* portion includes at least 50, 100 or 150 (contiguous) amino acid residues of an N-terminal half of a *hedgehog* protein. In preferred
15 embodiments, the *hedgehog* portion includes at least a portion of the *hedgehog* protein corresponding to a 19kd fragment of the extracellular domain of a *hedgehog* protein.

In preferred embodiments, the *hedgehog* portion has an amino acid sequence at least 60, 75, 85, or 95 percent identical with a *hedgehog* protein of any of SEQ ID Nos. 10-18 or 20, though sequences identical to those sequence listing entries are also contemplated as useful in the
20 present method. The *hedgehog* portion can be encoded by a nucleic acid which hybridizes under stringent conditions to a nucleic acid sequence of any of SEQ ID Nos. 1-9 or 19, e.g., the *hedgehog* portion can be encoded by a vertebrate *hedgehog* gene, especially a human *hedgehog* gene.

In other embodiments, the subject method can be carried out by administering a gene
25 activation construct, wherein the gene activation construct is designed to recombine with a genomic *hedgehog* gene of the patient to provide a heterologous transcriptional regulatory sequence operatively linked to a coding sequence of the *hedgehog* gene.

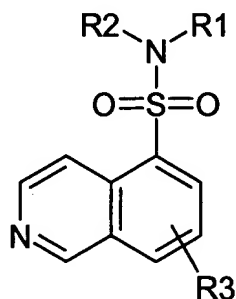
In still other embodiments, the subject method can be practiced with the administration of a gene therapy construct encoding a *hedgehog* polypeptide. For instance, the gene therapy
30 construct can be provided in a composition selected from a group consisting of a recombinant viral particle, a liposome, and a poly-cationic nucleic acid binding agent,

In yet other embodiments, the subject method can be carried out using a *ptc* therapeutic. An exemplary *ptc* therapeutic is a small organic molecule which binds to a *patched* protein and derepresses *patched*-mediated inhibition of mitosis, e.g., a molecule which binds to *patched* and mimics *hedgehog*-mediated *patched* signal transduction, which binds to *patched* and regulates *patched*-dependent gene expression. For instance, the binding of the *ptc* therapeutic to *patched* may result in upregulation of *patched* and/or *gli* expression.

In a more generic sense, the *ptc* therapeutic can be a small organic molecule which interacts with MK cells to induce *hedgehog*-mediated *patched* signal transduction, such as by altering the localization, protein-protein binding and/or enzymatic activity of an intracellular protein involved in a *patched* signal pathway. For instance, the *ptc* therapeutic may alter the level of expression of a *hedgehog* protein, a *patched* protein or a protein involved in the intracellular signal transduction pathway of *patched*.

In certain embodiments, the *ptc* therapeutic is an antisense construct which inhibits the expression of a protein which is involved in the signal transduction pathway of *patched* and the expression of which antagonizes *hedgehog*-mediated signals. The antisense construct is preferably an oligonucleotide of about 20-30 nucleotides in length and having a GC content of at least 50 percent.

In other embodiments, the *ptc* therapeutic is an inhibitor of protein kinase A (PKA), such as a 5-isoquinolinesulfonamide. The PKA inhibitor can be a cyclic AMP analog. Exemplary PKA inhibitors include N-[2-((p-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, 1-(5-isoquinoline-sulfonyl)-2-methylpiperazine, KT5720, 8-bromo-cAMP, dibutyryl-cAMP and PKA Heat Stable Inhibitor isoform α . Another exemplary PKA inhibitor is represented in the general formula:



wherein,

R_1 and R_2 each can independently represent hydrogen, and as valence and stability permit a lower alkyl, a lower alkenyl, a lower alkynyl, a carbonyl (such as a carboxyl, an ester, a formate, or a ketone), a thiocarbonyl (such as a thioester, a thioacetate, or a thioformate), an

amino, an acylamino, an amido, a cyano, a nitro, an azido, a sulfate, a sulfonate, a sulfonamido, $-(CH_2)_m-R_8$, $-(CH_2)_m-OH$, $-(CH_2)_m-O$ -lower alkyl, $-(CH_2)_m-O$ -lower alkenyl, $-(CH_2)_n-O-(CH_2)_m-R_8$, $-(CH_2)_m-SH$, $-(CH_2)_m-S$ -lower alkyl, $-(CH_2)_m-S$ -lower alkenyl, $-(CH_2)_n-S-(CH_2)_m-R_8$, or

5 R_1 and R_2 taken together with N form a heterocycle (substituted or unsubstituted);

R_3 is absent or represents one or more substitutions to the isoquinoline ring such as a lower alkyl, a lower alkenyl, a lower alkynyl, a carbonyl (such as a carboxyl, an ester, a formate, or a ketone), a thiocarbonyl (such as a thioester, a thioacetate, or a thioformate), an amino, an acylamino, an amido, a cyano, a nitro, an azido, a sulfate, a sulfonate, a sulfonamido, $-(CH_2)_m-R_8$, $-(CH_2)_m-OH$, $-(CH_2)_m-O$ -lower alkyl, $-(CH_2)_m-O$ -lower alkenyl, $-(CH_2)_n-O-(CH_2)_m-R_8$, $-(CH_2)_m-SH$, $-(CH_2)_m-S$ -lower alkyl, $-(CH_2)_m-S$ -lower alkenyl, $-(CH_2)_n-S-(CH_2)_m-R_8$;

R_8 represents a substituted or unsubstituted aryl, aralkyl, cycloalkyl, cycloalkenyl, or heterocycle; and

n and m are independently for each occurrence zero or an integer in the range of 1 to 6.

Brief Description of the Figures

Figures 1-4 illustrate the profile for staining with various markers for T-cell maturity.

Detailed Description of the Invention

I. Overview

The present application is directed to the discovery that hedgehog gene products are able to regulate maturation of T lymphocytes. Certain aspects of the invention are directed to preparations of hedgehog polypeptides, or other molecules which regulate *patched* or *smoothened* signalling, and their uses as immunomodulatory agents against both acquired and hereditary immunological disorders.

For instance, such compositions can be used to increase the population of T-helper cells to optimum levels in the host, e.g, to stimulate the immune system of the animal. Such uses of the subject compositions can be used in the treatment of bacterial or viral infections, as well as to help the body fight against cancer cells. Alternatively, these substances also enable the host to adjust to diseases arising from disarrangement of self-recognition processes in which there is excessive attack by host T-cells against endogenous tissues. In such instances, the subject

compositions can be used to reduce T-cell population so that the signs and symptoms of self-directed inflammatory (autoimmune) diseases such rheumatoid arthritis and multiple sclerosis are ameliorated.

As described herein, *hedgehog* proteins inhibit maturation of T lymphocytes. Based upon its inhibitory effect, the administration of *hedgehog* or *ptc* therapeutics (defined *infra*) is suggested herein as a treatment for several types of immunological disorders involving unwanted activation of cellular immunity, e.g., graft rejection, autoimmune disorders, and the like. In other embodiments, inhibitors of *hedgehog* signalling pathways can be used as immunostimulatory agents, e.g., to counteract the effects of any endogenous activation of a *hedgehog*/*ptc* pathway.

In general, the method of the present invention comprises administering to animal, or to cultured lymphocytes *in vitro*, an amount of a *hedgehog* or *ptc* therapeutic which produces a non-toxic response by the cell of inhibition of maturation (in the case of a *hedgehog* agonist) or promotion of T cell maturation (in the case of a *hedgehog* antagonist). The subject method can be carried out on cells which may be either dispersed in culture or a part of an intact tissue or organ. Moreover, the method can be performed on cells which are provided in culture (*in vitro*), or on cells in a whole animal (*in vivo*).

In one aspect, the present invention provides pharmaceutical preparations and methods for treating or preventing hyperimmune or hypoimmune disorders utilizing, as an active ingredient, a *hedgehog* polypeptide or a mimetic thereof. The invention also relates to methods of controlling the functional performance of T cells by use of the pharmaceutical preparations of the invention.

The subject *hedgehog* treatments are effective on both human and animal subjects afflicted with these conditions. Animal subjects to which the invention is applicable extend to both domestic animals and livestock, raised either as pets or for commercial purposes. Examples are dogs, cats, cattle, horses, sheep, hogs and goats.

Without wishing to be bound by any particular theory, the inhibitory effect of *hedgehog* on T cell maturation may be due at least in part to the ability of *hedgehog* proteins to antagonize (directly or indirectly) *patched*-mediated regulation of gene expression and other physiological effects mediated by that protein. The *patched* gene product, a cell surface protein, is understood to signal through a pathway which causes transcriptional repression of members of the Wnt and Dpp/BMP families of morphogens, proteins which impart positional information. In other tissue, the introduction of *hedgehog* relieves (derepresses) this inhibition conferred by *patched*, allowing expression of particular gene programs.

Recently, it has been reported that mutations in the human version of *patched*, a gene first identified in a fruit fly developmental pathway, cause a hereditary skin cancer and may contribute to sporadic skin cancers. See, for example, Hahn et al. (1996) *Cell* 86:841-851; and Johnson et al. (1996) *Science* 272:1668-1671. The demonstration that nevoid basal-cell carcinoma (NBCC) results from mutations in the human *patched* gene provided an example of the roles *patched* plays in post-embryonic development. These observations have led the art to understand one activity of *patched* to be a tumor suppressor gene, which may act by inhibiting proliferative signals from *hedgehog*. Our observations set forth below reveal potential new roles for the *hedgehog/patched* pathway in maintenance of mature T-cells and other lymphocytic cell lines. Accordingly, the present invention contemplates the use of other agents which are capable of mimicking or antagonizing, depending on the desired consequence, the effect of the *hedgehog* protein on *patched* signalling, e.g., as may be identified from the drug screening assays described below.

II. Definitions

For convenience, certain terms employed in the specification, examples, and appended claims are collected here.

The term "hedgehog therapeutic" refers to various forms of hedgehog polypeptides, as well as peptidomimetics, which can modulate the proliferation/differentiation state of lymphocytes, by, as will be clear from the context of individual examples, mimicking or potentiating (agonizing) or inhibiting (antagonizing) the effects of a naturally-occurring *hedgehog* protein. A *hedgehog* therapeutic which mimics or potentiates the activity of a wild-type hedgehog protein is a "hedgehog agonist". Conversely, a *hedgehog* therapeutic which inhibits the activity of a wild-type hedgehog protein is a "hedgehog antagonist".

In particular, the term "hedgehog polypeptide" encompasses preparations of *hedgehog* proteins and peptidyl fragments thereof, both agonist and antagonist forms as the specific context will make clear.

As used herein the term "bioactive fragment of a *hedgehog* protein" refers to a fragment of a full-length *hedgehog* polypeptide, wherein the fragment specifically agonizes or antagonizes inductive events mediated by wild-type *hedgehog* proteins. The *hedgehog* bioactive fragment preferably is a soluble extracellular portion of a *hedgehog* protein, where solubility is with reference to physiologically compatible solutions. Exemplary bioactive fragments are described in PCT publications WO 95/18856 and WO 96/17924.

The term "ptc therapeutic" refers to agents which either (i) mimic the effect of *hedgehog* proteins on *patched* signalling, e.g., which antagonize the cell-cycle inhibitory and/or T-cell maturation promoting activity of *patched*, or (ii) activate or potentiate *patched* signalling. In other embodiments, the ptc therapeutic can be a *hedgehog* antagonist. The ptc therapeutic can be, e.g., a peptide, a nucleic acid, a carbohydrate, a small organic molecule, or natural product extract (or fraction thereof).

An "effective amount" of, e.g., a *hedgehog* or ptc therapeutic, with respect to the subject method of treatment, refers to an amount of a composition, e.g., a *hedgehog* polypeptide, in a preparation which, when applied as part of a desired dosage regimen brings enhances or inhibits (as the case may be) T-cell maturation, relative to the absence of the *hedgehog* or ptc therapeutic, according to clinically acceptable standards for the disorder to be treated.

A "patient" or "subject" to be treated by the subject method can mean either a human or non-human animal.

The "growth state" of a cell refers to the rate of proliferation of the cell and the state of differentiation of the cell.

"Homology" and "identity" each refer to sequence similarity between two polypeptide sequences, with identity being a more strict comparison. Homology and identity can each be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When a position in the compared sequence is occupied by the same amino acid residue, then the polypeptides can be referred to as identical at that position; when the equivalent site is occupied by the same amino acid (e.g., identical) or a similar amino acid (e.g., similar in steric and/or electronic nature), then the molecules can be referred to as homologous at that position. A percentage of homology or identity between sequences is a function of the number of matching or homologous positions shared by the sequences. An "unrelated" or "non-homologous" sequence shares less than 40 percent identity, though preferably less than 25 percent identity, with an *hedgehog* sequence of the present invention.

The term "corresponds to", when referring to a particular polypeptide or nucleic acid sequence is meant to indicate that the sequence of interest is identical or homologous to the reference sequence to which it is said to correspond.

The terms "recombinant protein", "heterologous protein" and "exogenous protein" are used interchangeably throughout the specification and refer to a polypeptide which is produced by recombinant DNA techniques, wherein generally, DNA encoding the polypeptide is inserted

into a suitable expression construct which is in turn used to transform a host cell to produce the heterologous protein. That is, the polypeptide is expressed from a heterologous nucleic acid.

A "chimeric protein" or "fusion protein" is a fusion of a first amino acid sequence encoding a *hedgehog* polypeptide with a second amino acid sequence defining a domain foreign to and not substantially homologous with any domain of *hh* protein. A chimeric protein may present a foreign domain which is found (albeit in a different protein) in an organism which also expresses the first protein, or it may be an "interspecies", "intergenic", etc. fusion of protein structures expressed by different kinds of organisms. In general, a fusion protein can be represented by the general formula $(X)_n-(hh)_m-(Y)_n$, wherein *hh* represents all or a portion of the *hedgehog* protein, X and Y each independently represent an amino acid sequences which are not naturally found as a polypeptide chain contiguous with the *hedgehog* sequence, m is an integer greater than or equal to 1, and each occurrence of n is, independently, 0 or an integer greater than or equal to 1 (n and m are preferably no greater than 5 or 10).

The term "CD4⁺ lymphocyte" is intended to mean a lymphocyte having a plurality of cell surface CD4 molecules as evidenced by binding of an antibody specific for CD4 to the cell surface, e.g., monoclonal antibody OKT4 (Ortho Diagnostics, Piscataway, N.J.).

The term "CD8⁺ lymphocyte" is intended to mean a lymphocyte having a plurality of cell surface CD8 molecules as evidenced by binding of an antibody specific for CD8 to the cell surface, e.g., monoclonal antibody OKT8 (Ortho Diagnostics, Piscataway, N.J.).

"Peripheral blood leukocytes", abbreviated PBL, are intended to mean the cellular components of the immune system in blood, e.g., lymphocytes, monocytes, eosinophils, basophils, neutrophils, plasma cells, mast cell precursors, and the like.

"Lymphocytes" are intended to encompass T-lymphocytes, (also referred to as T-cells), B-lymphocytes (also referred to as B-cells), natural killer-lymphocytes (NK-cells), cytotoxic T lymphocytes (CTL), T-helper lymphocytes, delta gamma -T-cell receptor bearing cells as well as precursors and activated derivatives thereof

"Activated lymphocyte" is intended to mean that subset of lymphocytes which has been i) exposed to a stimulus, and ii) has been triggered to change from a metabolically-quiescent cell into a cell with increased protein synthesis and/or DNA and RNA synthesis and possibly cell division. Illustrative stimuli and triggering pathways leading to activated lymphocytes include interaction of T cell receptors with antigens, interaction of interleukin receptors with interleukins, interaction of growth factor receptors with growth factors, interaction of lymphocyte cell surface determinants with antibody (e.g., anti-CD2) or mitogens (e.g., PHA), and the like.

"Subject in need thereof" is intended to mean a mammal having one or more clinical or laboratory indicia of a disorder or disease. The subject may exhibit clinical disease activity or may have a subclinical or latent infection. Subjects in need thereof include human and non-human primates, mammals, domestic animals, livestock, and the like, e.g., dogs, cats, rodents, birds, horses, cows, pigs and fish.

III. Exemplary Applications of Method and Compositions

According to the invention, preparations with ptc and hedgehog therapeutics are used in immunoregulatory disorders and diseases in animals and humans, for the prevention or prophylaxis, control, diagnosis or treatment thereof.

The subject method has wide applicability to the treatment or prophylaxis of disorders affecting the regulation of lymphocytes, particularly maturation and/or activation of T lymphocytes. In general, the method can be characterized as including a step of administering to an animal an amount of a ptc or hedgehog therapeutic effective to alter the proliferative and/or differentiation state of treated lymphocytes. Such therapeutic compositions may be useful in treatments designed to modulate, e.g., increase or decrease, an immunological response. Such diseases and conditions include, but are not limited to, infection (such as bacterial or viral infection), metabolic disease such as diabetes, nutritional deficiency, toxic agents, graft rejection or other hyperacute response, or autoimmune disorders. The goals of treatment in each case can be twofold: (1) to eliminate the cause of the disease or unwanted immunological response, and (2) to relieve its symptoms.

In view of their immunosuppressant activity, the hedgehog proteins (and agonists thereof) are suitable for preventing and treating diseases and conditions which require a temporary or permanent reduction or suppression of an immune response. In particular, their use extends to suppressing the activation of the proliferation of lymphocytes or cytotoxic T-cells and/or immunocytes, e.g. for preventing or treating autoimmune diseases such as diseases of the rheumatic type, multiple sclerosis, psoriasis, atopic dermatitis, or for preventing the rejection of transplanted tissues or organs such as kidneys, heart, lungs, bone marrow, spleen, skin or cornea, in undesirable reactions during or after transfusions, allergic diseases, particularly those which affect the gastrointestinal tract and which may take the form of an inflammation, or inflammatory, proliferative and hyperproliferative diseases and cutaneous manifestations of immunological disorders such as eczematous dermatitis, urticaria, vasculitis and scleroderma.

Thus, it is particularly advantageous to use immunosuppressive forms of the subject hedgehog and ptc therapeutics clinically for the disorders, diseases and conditions described above, i.e. when it is desirable to achieve immunosuppression in an animal or human body.

Depending on the nature and cause of the disease or disorder to be treated or the condition which is to be influenced in an animal or human body, it may be desirable to administer the hedgehog or ptc therapeutic preparation systemically, locally or topically to the tissue or organ in question. Systemic action is desirable, for example, when various organs or organ systems are in need of treatment, as is the case for example in systemic autoimmune diseases or allergies or in transplants of large, foreign organs or tissues. By contrast, a local effect would be considered if only local manifestations of an immunological occurrence had to be treated, e.g. in small transplants of skin or cornea or in cases of local dermatitis.

Depending on the duration and intensity of the immunosuppressant activity required, the hedgehog or ptc therapeutic preparations may be given one or more times a day, as well as intermittently, over a period of several days, weeks or months and in various dosages.

In still other embodiments, antagonist (lymphocyte maturation promoting) forms of the subject ptc and hedgehog therapeutics can be used to treat disorders involving hyp immunity, e.g., immunosuppressed or immunocompromised patients. For instance, the effect of hedgehog-induced immunosuppression, e.g., resulting from endogenous or heterologous activation of the hedgehog signalling pathway, can be counteracted using antagonist forms of the subject ptc and hedgehog therapeutics.

Thus, the subject method contemplates the treatment of immunocompromised subjects to increase one or more indicia of cell mediated immunity (CMI), humoral immunity, or innate resistance to infection, by administering pharmaceutical preparations of an activating ptc or hedgehog therapeutic. In certain embodiments, such immunity-promoting activities of antagonists forms of the subject ptc and hedgehog therapeutics can be identified, e.g., by i) increased E-rosette forming cells (E-RFC) in thymocyte cultures after incubation with the subject ptc or hedgehog therapeutic agents; ii) increased E-RFC in cultures of thymocytes from aged animals after incubation with the subject ptc or hedgehog therapeutic agents; and, iii) increased expression of OKT 4⁺ in cultures of human peripheral blood T-lymphocytes from patients with secondary immunodeficiency syndromes following treatment with the subject ptc or hedgehog therapeutic agents. Increased expression of CD2 and CD4 accessory molecules on T-lymphocytes is compatible with a heightened state of innate or induced immunity to infection, e.g., by upregulating T-helper and cytotoxic T-lymphocytes to respond to lower levels of antigen.

Immunodeficiency states fall into three general etiologic categories. First, there is immunosuppression that occurs as a consequence of disease processes. Second, there are immunodeficiencies that arise because of therapy for other diseases, so-called iatrogenic immunodeficiencies. Third, immunodeficiencies may result from direct attack of T-lymphocytes by the human immunodeficiency virus (HIV) that causes the acquired immunodeficiency syndrome (AIDS).

Common disease processes that lead to immunodeficiency are malnutrition, neoplasias, aging, and infections. Malnourished people, patients with advanced widespread cancers and people with debilitating illnesses become sick and die more often because impaired cell-mediated and humoral immune responses increase susceptibility to infections by a variety of organisms. A state of generalized deficiency in immune responses is called anergy. Various types of infections, especially viral infections, lead to immunosuppression. A drug, such as a maturation-promoting form of a Ptc or hedgehog therapeutic, e.g., capable of making the T-helper lymphocyte components of the immune system more robust, will be an important therapeutic agent for increasing the resistance of the patient to infections. For example, Ptc or hedgehog therapeutic or its analogs, may be:

- administered to patients, especially older patients, before or just after admissions to hospitals in order to reduce the risks of nosocomial (hospital-induced) infections, a common and severe clinical problem

- administered to burn victims, because such individuals are especially prone to infections

- administered to patients in anticipation of epidemic infections, for example, in conjunction with influenza vaccinations or hepatitis vaccinations, to invigorate the immune response to pathogens

- administered to patients with asymptomatic viral infections, in order to enhance immune surveillance of pathogenic organisms and reduce the likelihood of recurrence of disease, for example, for individuals who are carriers of herpes viruses, varicella viruses, hepatitis viruses and HIV.

Iatrogenic immunosuppression is most often due to drug therapies which either kill or functionally inactivate lymphocytes. Various chemotherapeutic drugs are administered to cancer patients, and these drugs are usually cytotoxic to both mature and developing lymphocytes as well as to granulocyte and monocyte precursors. Thus, cancer chemotherapy is almost always accompanied by a period of immunosuppression and increased risk of infections. Radiation treatment of cancer carries the same risks. Medications (granulocyte-colony stimulating factor)

exist for increasing neutrophils in blood to combat infections that occur after cancer chemotherapy, but no medications are currently used for restoring lymphocytic functions. Major surgery, for example repair of aneurysms or by-pass operations, also decrease immune function in humans. The reasons for the decline in blood lymphocytes that occur because of major surgery are not clear, but an agent that elevates lymphocyte functions in such patients have therapeutic value in decreasing the likelihood of infections.

One final form of acquired immunosuppression that should be mentioned results from the absence of a spleen, caused by surgical removal of the organ after trauma or for the treatment of certain hematologic diseases or as a result of infarction in sickle cell disease. Patients without spleens are more susceptible to infections by some organisms, particularly encapsulated bacteria such as *Streptococcus pneumoniae*. The spleen is apparently required for the induction of protective humoral immune responses to such organisms. The subject Ptc or hedgehog therapeutics can help individuals without a spleen or without a thymus in resistance against infection by micro-organisms.

IV. Exemplary hedgehog therapeutic compounds.

The *hedgehog* therapeutic compositions of the subject method can be generated by any of a variety of techniques, including purification of naturally occurring proteins, recombinantly produced proteins and synthetic chemistry. Polypeptide forms of the hedgehog therapeutics are preferably derived from vertebrate hedgehog proteins, e.g., have sequences corresponding to naturally occurring hedgehog proteins, or fragments thereof, from vertebrate organisms. However, it will be appreciated that the hedgehog polypeptide can correspond to a hedgehog protein (or fragment thereof) which occurs in any metazoan organism.

The various naturally-occurring *hedgehog* proteins from which the subject therapeutics can be derived are characterized by a signal peptide, a highly conserved N-terminal region, and a more divergent C-terminal domain. In addition to signal sequence cleavage in the secretory pathway (Lee, J.J. *et al.* (1992) *Cell* 71:33-50; Tabata, T. *et al.* (1992) *Genes Dev.* 2635-2645; Chang, D.E. *et al.* (1994) *Development* 120:3339-3353), *hedgehog* precursor proteins naturally undergo an internal autoproteolytic cleavage which depends on conserved sequences in the C-terminal portion (Lee *et al.* (1994) *Science* 266:1528-1537; Porter *et al.* (1995) *Nature* 374:363-366). This autocleavage leads to a 19 kD N-terminal peptide and a C-terminal peptide of 26-28 kD (Lee *et al.* (1992) *supra*; Tabata *et al.* (1992) *supra*; Chang *et al.* (1994) *supra*; Lee *et al.* (1994) *supra*; Bumcrot, D.A., *et al.* (1995) *Mol. Cell. Biol.* 15:2294-2303; Porter *et al.* (1995) *supra*; Ekker, S.C. *et al.* (1995) *Curr. Biol.* 5:944-955; Lai, C.J. *et al.* (1995) *Development*

121:2349-2360). The N-terminal peptide stays tightly associated with the surface of cells in which it was synthesized, while the C-terminal peptide is freely diffusible both *in vitro* and *in vivo* (Lee *et al.* (1994) *supra*; Bumcrot *et al.* (1995) *supra*; Mart', E. *et al.* (1995) *Development* 121:2537-2547; Roelink, H. *et al.* (1995) *Cell* 81:445-455). Cell surface retention of the N-terminal peptide is dependent on autocleavage, as a truncated form of *hedgehog* encoded by an RNA which terminates precisely at the normal position of internal cleavage is diffusible *in vitro* (Porter *et al.* (1995) *supra*) and *in vivo* (Porter, J.A. *et al.* (1996) *Cell* 86, 21-34). Biochemical studies have shown that the autoproteolytic cleavage of the *hedgehog* precursor protein proceeds through an internal thioester intermediate which subsequently is cleaved in a nucleophilic substitution. It is suggested that the nucleophile is a small lipophilic molecule, more particularly cholesterol, which becomes covalently bound to the C-terminal end of the N-peptide (Porter *et al.* (1996) *supra*), tethering it to the cell surface.

The vertebrate family of *hedgehog* genes includes at least four members, e.g., paralogs of the single drosophila *hedgehog* gene (SEQ ID No. 19). Three of these members, herein referred to as Desert *hedgehog* (*Dhh*), Sonic *hedgehog* (*Shh*) and Indian *hedgehog* (*Ihh*), apparently exist in all vertebrates, including fish, birds, and mammals. A fourth member, herein referred to as tiggie-winkle *hedgehog* (*Thh*), appears specific to fish. According to the appended sequence listing, (see also Table 1) a chicken *Shh* polypeptide is encoded by SEQ ID No:1; a mouse *Dhh* polypeptide is encoded by SEQ ID No:2; a mouse *Ihh* polypeptide is encoded by SEQ ID No:3; a mouse *Shh* polypeptide is encoded by SEQ ID No:4 a zebrafish *Shh* polypeptide is encoded by SEQ ID No:5; a human *Shh* polypeptide is encoded by SEQ ID No:6; a human *Ihh* polypeptide is encoded by SEQ ID No:7; a human *Dhh* polypeptide is encoded by SEQ ID No. 8; and a zebrafish *Thh* is encoded by SEQ ID No. 9.

Table 1
Guide to *hedgehog* sequences in Sequence Listing

	Nucleotide	Amino Acid
Chicken <i>Shh</i>	SEQ ID No. 1	SEQ ID No. 10
Mouse <i>Dhh</i>	SEQ ID No. 2	SEQ ID No. 11
Mouse <i>Ihh</i>	SEQ ID No. 3	SEQ ID No. 12
Mouse <i>Shh</i>	SEQ ID No. 4	SEQ ID No. 13
Zebrafish <i>Shh</i>	SEQ ID No. 5	SEQ ID No. 14
Human <i>Shh</i>	SEQ ID No. 6	SEQ ID No. 15
Human <i>Ihh</i>	SEQ ID No. 7	SEQ ID No. 16
Human <i>Dhh</i>	SEQ ID No. 8	SEQ ID No. 17
Zebrafish <i>Thh</i>	SEQ ID No. 9	SEQ ID No. 18
Drosophila <i>HH</i>	SEQ ID No. 19	SEQ ID No. 20

In addition to the sequence variation between the various *hedgehog* homologs, the *hedgehog* proteins are apparently present naturally in a number of different forms, including a pro-form, a full-length mature form, and several processed fragments thereof. The pro-form includes an N-terminal signal peptide for directed secretion of the extracellular domain, while the full-length mature form lacks this signal sequence.

As described above, further processing of the mature form occurs in some instances to yield biologically active fragments of the protein. For instance, *sonic hedgehog* undergoes additional proteolytic processing to yield two peptides of approximately 19 kDa and 27 kDa, the 19kDa fragment corresponding to an proteolytic N-terminal portion of the mature protein.

In addition to proteolytic fragmentation, the vertebrate *hedgehog* proteins can also be modified post-translationally, such as by glycosylation and/or addition of lipophilic moieties, such as stents, fatty acids, etc., though bacterially produced (e.g. unmodified) forms of the proteins still maintain certain of the bioactivities of the native protein. Bioactive fragments of *hedgehog* polypeptides of the present invention have been generated and are described in great detail in, e.g., PCT publications WO 95/18856 and WO 96/17924.

There are a wide range of lipophilic moieties with which *hedgehog* polypeptides can be derivatized. The term "lipophilic group", in the context of being attached to a *hedgehog* polypeptide, refers to a group having high hydrocarbon content thereby giving the group high affinity to lipid phases. A lipophilic group can be, for example, a relatively long chain alkyl or cycloalkyl (preferably n-alkyl) group having approximately 7 to 30 carbons. The alkyl group may terminate with a hydroxy or primary amine "tail". To further illustrate, lipophilic molecules include naturally-occurring and synthetic aromatic and non-aromatic moieties such as fatty acids, sterols, esters and alcohols, other lipid molecules, cage structures such as adamantane and buckminsterfullerenes, and aromatic hydrocarbons such as benzene, perylene, phenanthrene, anthracene, naphthalene, pyrene, chrysene, and naphthacene.

In one embodiment, the *hedgehog* polypeptide is modified with one or more sterol moieties, such as cholesterol. See, for example, PCT publication WO 96/17924. In certain embodiments, the cholesterol is preferably added to the C-terminal glycine where the *hedgehog* polypeptide corresponds to the naturally-occurring N-terminal proteolytic fragment.

In another embodiment, the *hedgehog* polypeptide can be modified with a fatty acid moiety, such as a myristoyl, palmitoyl, stearoyl, or arachidoyl moiety. See, e.g., Pepinsky et al. (1998) J Biol. Chem 273: 14037.

In addition to those effects seen by cholesterol-addition to the C-terminus or fatty acid addition to the N-terminus of extracellular fragments of the protein, at least certain of the biological activities of the hedgehog gene products are unexpectedly potentiated by derivatization of the protein with lipophilic moieties at other sites on the protein and/or by moieties other than cholesterol or fatty acids. Certain aspects of the invention are directed to the use of preparations of hedgehog polypeptides which are modified at sites other than N-terminal or C-terminal residues of the natural processed form of the protein, and/or which are modified at such terminal residues with lipophilic moieties other than a sterol at the C-terminus or fatty acid at the N-terminus.

Particularly useful as lipophilic molecules are alicyclic hydrocarbons, saturated and unsaturated fatty acids and other lipid and phospholipid moieties, waxes, cholesterol, isoprenoids, terpenes and polyalicyclic hydrocarbons including adamantane and buckminsterfullerenes, vitamins, polyethylene glycol or oligoethylene glycol, (C1-C18)-alkyl phosphate diesters, -O-CH₂-CH(OH)-O-(C12-C18)-alkyl, and in particular conjugates with pyrene derivatives. The lipophilic moiety can be a lipophilic dye suitable for use in the invention include, but are not limited to, diphenylhexatriene, Nile Red, N-phenyl-1-naphthylamine, Prodan, Laurodan, Pyrene, Perylene, rhodamine, rhodamine B, tetramethylrhodamine, Texas Red, sulforhodamine, 1,1'-didodecyl-3,3,3',3'tetramethylindocarbocyanine perchlorate, octadecyl rhodamine B and the BODIPY dyes available from Molecular Probes Inc.

Other exemplary lipophilic moieties include aliphatic carbonyl radical groups include 1- or 2-adamantylacetyl, 3-methyladamant-1-ylacetyl, 3-methyl-3-bromo-1-adamantylacetyl, 1-decalinacetyl, camphoracetyl, camphaneacetyl, noradamantylacetyl, norbornaneacetyl, bicyclo[2.2.2.]-oct-5-eneacetyl, 1-methoxybicyclo[2.2.2.]-oct-5-ene-2-carbonyl, cis-5-norbornene-endo-2,3-dicarbonyl, 5-norbornene-2-ylacetyl, (1R)-(-)-myrtentaneacetyl, 2-norbornaneacetyl, anti-3-oxo-tricyclo[2.2.1.0<2,6>]-heptane-7-carbonyl, decanoyl, dodecanoyl, dodecenoyl, tetradecadienoyl, decynoyl or dodecynoyl.

The hedgehog polypeptide can be linked to the hydrophobic moiety in a number of ways including by chemical coupling means, or by genetic engineering.

There are a large number of chemical cross-linking agents that are known to those skilled in the art. For the present invention, the preferred cross-linking agents are heterobifunctional cross-linkers, which can be used to link the hedgehog polypeptide and hydrophobic moiety in a stepwise manner. Heterobifunctional cross-linkers provide the ability to design more specific coupling methods for conjugating to proteins, thereby reducing the occurrences of unwanted side reactions such as homo-protein polymers. A wide variety of heterobifunctional cross-linkers are

known in the art. These include: succinimidyl 4-(N-maleimidomethyl) cyclohexane- 1-carboxylate (SMCC), m-Maleimidobenzoyl-N- hydroxysuccinimide ester (MBS); N-succinimidyl (4-iodoacetyl) aminobenzoate (SIAB), succinimidyl 4-(p-maleimidophenyl) butyrate (SMPB), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC); 4-succinimidylloxycarbonyl- a-methyl-a-(2-pyridyldithio)-tolune (SMPT), N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP), succinimidyl 6-[3-(2-pyridyldithio) propionate] hexanoate (LC-SPDP). Those cross-linking agents having N-hydroxysuccinimide moieties can be obtained as the N-hydroxysulfosuccinimide analogs, which generally have greater water solubility. In addition, those cross-linking agents having disulfide bridges within the linking chain can be synthesized instead as the alkyl derivatives so as to reduce the amount of linker cleavage *in vivo*.

In addition to the heterobifunctional cross-linkers, there exists a number of other cross-linking agents including homobifunctional and photoreactive cross-linkers. Disuccinimidyl suberate (DSS), bismaleimido-hexane (BMH) and dimethylpimelimidate-2 HCl (DMP) are examples of useful homobifunctional cross-linking agents, and bis-[β -(4-azidosalicylamido)ethyl]disulfide (BASED) and N-succinimidyl-6(4'-azido-2'-nitrophenyl-amino)hexanoate (SANPAH) are examples of useful photoreactive cross-linkers for use in this invention. For a recent review of protein coupling techniques, see Means et al. (1990) *Bioconjugate Chemistry* 1:2-12, incorporated by reference herein.

One particularly useful class of heterobifunctional cross-linkers, included above, contain the primary amine reactive group, N-hydroxysuccinimide (NHS), or its water soluble analog N-hydroxysulfosuccinimide (sulfo-NHS). Primary amines (lysine epsilon groups) at alkaline pH's are unprotonated and react by nucleophilic attack on NHS or sulfo-NHS esters. This reaction results in the formation of an amide bond, and release of NHS or sulfo-NHS as a by-product.

Another reactive group useful as part of a heterobifunctional cross-linker is a thiol reactive group. Common thiol reactive groups include maleimides, halogens, and pyridyl disulfides. Maleimides react specifically with free sulfhydryls (cysteine residues) in minutes, under slightly acidic to neutral (pH 6.5-7.5) conditions. Halogens (iodoacetyl functions) react with -SH groups at physiological pH's. Both of these reactive groups result in the formation of stable thioether bonds.

The third component of the heterobifunctional cross-linker is the spacer arm or bridge. The bridge is the structure that connects the two reactive ends. The most apparent attribute of the bridge is its effect on steric hindrance. In some instances, a longer bridge can more easily span the distance necessary to link two complex biomolecules. For instance, SMPB has a span of 14.5 angstroms.

Preparing protein-protein conjugates using heterobifunctional reagents is a two-step process involving the amine reaction and the sulfhydryl reaction. For the first step, the amine reaction, the protein chosen should contain a primary amine. This can be lysine epsilon amines or a primary alpha amine found at the N-terminus of most proteins. The protein should not contain free sulfhydryl groups. In cases where both proteins to be conjugated contain free sulfhydryl groups, one protein can be modified so that all sulfhydryls are blocked using for instance, N-ethylmaleimide (see Partis et al. (1983) J. Pro. Chem. 2:263, incorporated by reference herein). Ellman's Reagent can be used to calculate the quantity of sulfhydryls in a particular protein (see for example Ellman et al. (1958) Arch. Biochem. Biophys. 74:443 and Riddles et al. (1979) Anal. Biochem. 94:75, incorporated by reference herein).

The reaction buffer should be free of extraneous amines and sulfhydryls. The pH of the reaction buffer should be 7.0-7.5. This pH range prevents maleimide groups from reacting with amines, preserving the maleimide group for the second reaction with sulfhydryls.

The NHS-ester containing cross-linkers have limited water solubility. They should be dissolved in a minimal amount of organic solvent (DMF or DMSO) before introducing the cross-linker into the reaction mixture. The cross-linker/solvent forms an emulsion which will allow the reaction to occur.

The sulfo-NHS ester analogs are more water soluble, and can be added directly to the reaction buffer. Buffers of high ionic strength should be avoided, as they have a tendency to "salt out" the sulfo-NHS esters. To avoid loss of reactivity due to hydrolysis, the cross-linker is added to the reaction mixture immediately after dissolving the protein solution.

The reactions can be more efficient in concentrated protein solutions. The more alkaline the pH of the reaction mixture, the faster the rate of reaction. The rate of hydrolysis of the NHS and sulfo-NHS esters will also increase with increasing pH. Higher temperatures will increase the reaction rates for both hydrolysis and acylation.

Once the reaction is completed, the first protein is now activated, with a sulfhydryl reactive moiety. The activated protein may be isolated from the reaction mixture by simple gel filtration or dialysis. To carry out the second step of the cross-linking, the sulfhydryl reaction, the lipophilic group chosen for reaction with maleimides, activated halogens, or pyridyl disulfides must contain a free sulfhydryl. Alternatively, a primary amine may be modified with to add a sulfhydryl

In all cases, the buffer should be degassed to prevent oxidation of sulfhydryl groups. EDTA may be added to chelate any oxidizing metals that may be present in the buffer. Buffers should be free of any sulfhydryl containing compounds.

Maleimides react specifically with -SH groups at slightly acidic to neutral pH ranges (6.5-7.5). A neutral pH is sufficient for reactions involving halogens and pyridyl disulfides. Under these conditions, maleimides generally react with -SH groups within a matter of minutes. Longer reaction times are required for halogens and pyridyl disulfides.

The first sulfhydryl reactive-protein prepared in the amine reaction step is mixed with the sulfhydryl-containing lipophilic group under the appropriate buffer conditions. The conjugates can be isolated from the reaction mixture by methods such as gel filtration or by dialysis.

Exemplary activated lipophilic moieties for conjugation include: N-(1-pyrene)maleimide; 2,5-dimethoxystilbene-4'-maleimide, eosin-5-maleimide; fluorescein-5-maleimide; N-(4-(6-dimethylamino-2-benzofuranyl)phenyl)maleimide; benzophenone-4-maleimide; 4-dimethylaminophenylazophenyl-4'-maleimide (DABMI), tetramethylrhodamine-5-maleimide, tetramethylrhodamine-6-maleimide, Rhodamine Red™ C2 maleimide, N-(5-aminopentyl)maleimide, trifluoroacetic acid salt, N-(2-aminoethyl)maleimide, trifluoroacetic acid salt, Oregon Green™ 488 maleimide, N-(2-(((4-azido-2,3,5,6-tetrafluoro)benzoyl)amino)ethyl)dithio)ethyl)maleimide (TFPAM-SS1), 2-(1-(3-dimethylaminopropyl)-indol-3-yl)-3-(indol-3-yl) maleimide (bisindolylmaleimide; GF 109203X), BODIPY® FL N-(2-aminoethyl)maleimide, N-(7-dimethylamino-4-methylcoumarin-3-yl)maleimide (DACM), Alexa™ 488 C5 maleimide, Alexa™ 594 C5 maleimide, sodium salt N-(1-pyrene)maleimide, 2,5-dimethoxystilbene-4'-maleimide, eosin-5-maleimide, fluorescein-5-maleimide, N-(4-(6-dimethylamino-2-benzofuranyl)phenyl)maleimide, benzophenone-4-maleimide, 4-dimethylaminophenylazophenyl-4'-maleimide, 1-(2-maleimidylethyl)-4-(5-(4-methoxyphenyl)oxazol-2-yl)pyridinium methanesulfonate, tetramethylrhodamine-5-maleimide, tetramethylrhodamine-6-maleimide, Rhodamine Red™ C2 maleimide, N-(5-aminopentyl)maleimide, N-(2-aminoethyl)maleimide, N-(2-(((4-azido-2,3,5,6-tetrafluoro)benzoyl)amino)ethyl)dithio)ethyl)maleimide, 2-(1-(3-dimethylaminopropyl)-indol-3-yl)-3-(indol-3-yl) maleimide, N-(7-dimethylamino-4-methylcoumarin-3-yl)maleimide (DACM), 11H-Benzo[a]fluorene, Benzo[a]pyrene.

In one embodiment, the hedgehog polypeptide can be derivatized using pyrene maleimide, which can be purchased from Molecular Probes (Eugene, Oreg.), e.g., N-(1-pyrene)maleimide or 1-pyrenemethyl iodoacetate (PMIA ester).

For those embodiments wherein the hydrophobic moiety is a polypeptide, the modified hedgehog polypeptide of this invention can be constructed as a fusion protein, containing the hedgehog polypeptide and the hydrophobic moiety as one contiguous polypeptide chain.

In certain embodiments, the lipophilic moiety is an amphipathic polypeptide, such as magainin, cecropin, attacin, melittin, gramicidin S, alpha-toxin of *Staph. aureus*, alamethicin or a synthetic amphipathic polypeptide. Fusogenic coat proteins from viral particles can also be a convenient source of amphipathic sequences for the subject hedgehog proteins

Moreover, mutagenesis can be used to create modified *hh* polypeptides, e.g., for such purposes as enhancing therapeutic or prophylactic efficacy, or stability (e.g., *ex vivo* shelf life and resistance to proteolytic degradation *in vivo*). Such modified peptides can be produced, for instance, by amino acid substitution, deletion, or addition. Modified *hedgehog* polypeptides can also include those with altered post-translational processing relative to a naturally occurring *hedgehog* protein, e.g., altered glycosylation, cholesterolization, prenylation and the like.

In one embodiment, the hedgehog therapeutic is a polypeptide encodable by a nucleotide sequence that hybridizes under stringent conditions to a hedgehog coding sequence represented in one or more of SEQ ID Nos:1-7. Appropriate stringency conditions which promote DNA hybridization, for example, 6.0 x sodium chloride/sodium citrate (SSC) at about 45°C, followed by a wash of 2.0 x SSC at 50°C, are known to those skilled in the art or can be found in *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. For example, the salt concentration in the wash step can be selected from a low stringency of about 2.0 x SSC at 50°C to a high stringency of about 0.2 x SSC at 50°C. In addition, the temperature in the wash step can be increased from low stringency conditions at room temperature, about 22°C, to high stringency conditions at about 65°C.

As described in the literature, genes for other hedgehog proteins, e.g., from other animals, can be obtained from mRNA or genomic DNA samples using techniques well known in the art. For example, a cDNA encoding a *hedgehog* protein can be obtained by isolating total mRNA from a cell, e.g. a mammalian cell, e.g. a human cell, including embryonic cells. Double stranded cDNAs can then be prepared from the total mRNA, and subsequently inserted into a suitable plasmid or bacteriophage vector using any one of a number of known techniques. The gene encoding a *hedgehog* protein can also be cloned using established polymerase chain reaction techniques.

Preferred nucleic acids encode a *hedgehog* polypeptide comprising an amino acid sequence at least 60% homologous or identical, more preferably 70% homologous or identical, and most preferably 80% homologous or identical with an amino acid sequence selected from the

group consisting of SEQ ID Nos:8-14. Nucleic acids which encode polypeptides at least about 90%, more preferably at least about 95%, and most preferably at least about 98-99% homology or identity with an amino acid sequence represented in one of SEQ ID Nos:8-14 are also within the scope of the invention.

5 In addition to native *hedgehog* proteins, *hedgehog* polypeptides preferred by the present invention are at least 60% homologous or identical, more preferably 70% homologous or identical and most preferably 80% homologous or identical with an amino acid sequence represented by any of SEQ ID Nos:8-14. Polypeptides which are at least 90%, more preferably at least 95%, and most preferably at least about 98-99% homologous or identical with a sequence
10 selected from the group consisting of SEQ ID Nos:8-14 are also within the scope of the invention. The only prerequisite is that the *hedgehog* polypeptide is capable of modulating the growth state of T lymphocytes.

The term "recombinant protein" refers to a polypeptide of the present invention which is produced by recombinant DNA techniques, wherein generally, DNA encoding a *hedgehog* polypeptide is inserted into a suitable expression vector which is in turn used to transform a host cell to produce the heterologous protein. Moreover, the phrase "derived from", with respect to a recombinant *hedgehog* gene, is meant to include within the meaning of "recombinant protein" those proteins having an amino acid sequence of a native *hedgehog* protein, or an amino acid sequence similar thereto which is generated by mutations including substitutions and deletions
15 (including truncation) of a naturally occurring form of the protein.

The method of the present invention can also be carried out using variant forms of the naturally occurring *hedgehog* polypeptides, e.g., mutational variants.

As is known in the art, *hedgehog* polypeptides can be produced by standard biological techniques or by chemical synthesis. For example, a host cell transfected with a nucleic acid vector directing expression of a nucleotide sequence encoding the subject polypeptides can be
25 cultured under appropriate conditions to allow expression of the peptide to occur. The polypeptide *hedgehog* may be secreted and isolated from a mixture of cells and medium containing the recombinant *hedgehog* polypeptide. Alternatively, the peptide may be retained cytoplasmically by removing the signal peptide sequence from the recombinant *hedgehog* gene and the cells harvested, lysed and the protein isolated. A cell culture includes host cells, media and other byproducts. Suitable media for cell culture are well known in the art. The recombinant
30 *hedgehog* polypeptide can be isolated from cell culture medium, host cells, or both using techniques known in the art for purifying proteins including ion-exchange chromatography, gel filtration chromatography, ultrafiltration, electrophoresis, and immunoaffinity purification with

antibodies specific for such peptide. In a preferred embodiment, the recombinant *hedgehog* polypeptide is a fusion protein containing a domain which facilitates its purification, such as an *hedgehog*/GST fusion protein. The host cell may be any prokaryotic or eukaryotic cell.

Recombinant *hedgehog* genes can be produced by ligating nucleic acid encoding an *hedgehog* protein, or a portion thereof, into a vector suitable for expression in either prokaryotic cells, eukaryotic cells, or both. Expression vectors for production of recombinant forms of the subject *hedgehog* polypeptides include plasmids and other vectors. For instance, suitable vectors for the expression of a *hedgehog* polypeptide include plasmids of the types: pBR322-derived plasmids, pEMBL-derived plasmids, pEX-derived plasmids, pBTac-derived plasmids and pUC-derived plasmids for expression in prokaryotic cells, such as *E. coli*.

A number of vectors exist for the expression of recombinant proteins in yeast. For instance, YEP24, YIP5, YEP51, YEP52, pYES2, and YRP17 are cloning and expression vehicles useful in the introduction of genetic constructs into *S. cerevisiae* (see, for example, Broach *et al.* (1983) in *Experimental Manipulation of Gene Expression*, ed. M. Inouye Academic Press, p. 83, incorporated by reference herein). These vectors can replicate in *E. coli* due to the presence of the pBR322 ori, and in *S. cerevisiae* due to the replication determinant of the yeast 2 micron plasmid. In addition, drug resistance markers such as ampicillin can be used. In an illustrative embodiment, an *hedgehog* polypeptide is produced recombinantly utilizing an expression vector generated by sub-cloning the coding sequence of one of the *hedgehog* genes represented in SEQ ID Nos:1-7.

The preferred mammalian expression vectors contain both prokaryotic sequences, to facilitate the propagation of the vector in bacteria, and one or more eukaryotic transcription units that are expressed in eukaryotic cells. The pcDNAI/amp, pcDNAI/neo, pRc/CMV, pSV2gpt, pSV2neo, pSV2-dhfr, pTk2, pRSVneo, pMSG, pSVT7, pko-neo and pHyg derived vectors are examples of mammalian expression vectors suitable for transfection of eukaryotic cells. Some of these vectors are modified with sequences from bacterial plasmids, such as pBR322, to facilitate replication and drug resistance selection in both prokaryotic and eukaryotic cells. Alternatively, derivatives of viruses such as the bovine papillomavirus (BPV-1), or Epstein-Barr virus (pHEBo, pREP-derived and p205) can be used for transient expression of proteins in eukaryotic cells. The various methods employed in the preparation of the plasmids and transformation of host organisms are well known in the art. For other suitable expression systems for both prokaryotic and eukaryotic cells, as well as general recombinant procedures, see *Molecular Cloning A Laboratory Manual*, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989) Chapters 16 and 17.

In some instances, it may be desirable to express the recombinant *hedgehog* polypeptide by the use of a baculovirus expression system. Examples of such baculovirus expression systems include pVL-derived vectors (such as pVL1392, pVL1393 and pVL941), pAcUW-derived vectors (such as pAcUW1), and pBlueBac-derived vectors (such as the β -gal containing pBlueBac III).

When it is desirable to express only a portion of an *hedgehog* protein, such as a form lacking a portion of the N-terminus, i.e. a truncation mutant which lacks the signal peptide, it may be necessary to add a start codon (ATG) to the oligonucleotide fragment containing the desired sequence to be expressed. It is well known in the art that a methionine at the N-terminal position can be enzymatically cleaved by the use of the enzyme methionine aminopeptidase (MAP). MAP has been cloned from *E. coli* (Ben-Bassat et al. (1987) *J. Bacteriol.* 169:751-757) and *Salmonella typhimurium* and its *in vitro* activity has been demonstrated on recombinant proteins (Miller et al. (1987) *PNAS* 84:2718-1722). Therefore, removal of an N-terminal methionine, if desired, can be achieved either *in vivo* by expressing *hedgehog*-derived polypeptides in a host which produces MAP (e.g., *E. coli* or CM89 or *S. cerevisiae*), or *in vitro* by use of purified MAP (e.g., procedure of Miller et al., *supra*).

Alternatively, the coding sequences for the polypeptide can be incorporated as a part of a fusion gene including a nucleotide sequence encoding a different polypeptide. It is widely appreciated that fusion proteins can also facilitate the expression of proteins, and accordingly, can be used in the expression of the *hedgehog* polypeptides of the present invention. For example, *hedgehog* polypeptides can be generated as glutathione-S-transferase (GST-fusion) proteins. Such GST-fusion proteins can enable easy purification of the *hedgehog* polypeptide, as for example by the use of glutathione-derivatized matrices (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel et al. (N.Y.: John Wiley & Sons, 1991)). In another embodiment, a fusion gene coding for a purification leader sequence, such as a poly-(His)/enterokinase cleavage site sequence, can be used to replace the signal sequence which naturally occurs at the N-terminus of the *hedgehog* protein (e.g. of the pro-form, in order to permit purification of the poly(His)-*hedgehog* protein by affinity chromatography using a Ni^{2+} metal resin. The purification leader sequence can then be subsequently removed by treatment with enterokinase (e.g., see Hochuli et al. (1987) *J. Chromatography* 411:177; and Janknecht et al. *PNAS* 88:8972).

Techniques for making fusion genes are known to those skilled in the art. Essentially, the joining of various DNA fragments coding for different polypeptide sequences is performed in accordance with conventional techniques, employing blunt-ended or stagger-ended termini for

ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed to generate a chimeric gene sequence (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel et al. John Wiley & Sons: 1992).

Hedgehog polypeptides may also be chemically modified to create *hedgehog* derivatives by forming covalent or aggregate conjugates with other chemical moieties, such as glycosyl groups, cholesterol, isoprenoids, lipids, phosphate, acetyl groups and the like. Covalent derivatives of *hedgehog* proteins can be prepared by linking the chemical moieties to functional groups on amino acid sidechains of the protein or at the N-terminus or at the C-terminus of the polypeptide.

For instance, *hedgehog* proteins can be generated to include a moiety, other than sequence naturally associated with the protein, that binds a component of the extracellular matrix and enhances localization of the analog to cell surfaces. For example, sequences derived from the fibronectin "type-III repeat", such as a tetrapeptide sequence R-G-D-S (Pierschbacher et al. (1984) *Nature* 309:30-3; and Kornblihtt et al. (1985) *EMBO* 4:1755-9) can be added to the *hedgehog* polypeptide to support attachment of the chimeric molecule to a cell through binding ECM components (Ruoslahti et al. (1987) *Science* 238:491-497; Pierschbacher et al. (1987) *J. Biol. Chem.* 262:17294-8.; Hynes (1987) *Cell* 48:549-54; and Hynes (1992) *Cell* 69:11-25).

In a preferred embodiment, the *hedgehog* polypeptide is isolated from, or is otherwise substantially free of, other cellular proteins, especially other extracellular or cell surface associated proteins which may normally be associated with the *hedgehog* polypeptide, unless provided in the form of fusion protein with the *hedgehog* polypeptide. The term "substantially free of other cellular or extracellular proteins" (also referred to herein as "contaminating proteins") or "substantially pure preparations" or "purified preparations" are defined as encompassing preparations of *hedgehog* polypeptides having less than 20% (by dry weight) contaminating protein, and preferably having less than 5% contaminating protein. By "purified", it is meant that the indicated molecule is present in the substantial absence of other biological macromolecules, such as other proteins. The term "purified" as used herein preferably means at least 80% by dry weight, more preferably in the range of 95-99% by weight, and most preferably at least 99.8% by weight, of biological macromolecules of the same type present (but water,

buffers, and other small molecules, especially molecules having a molecular weight of less than 5000, can be present). The term "pure" as used herein preferably has the same numerical limits as "purified" immediately above.

As described above for recombinant polypeptides, isolated *hedgehog* polypeptides can include all or a portion of the amino acid sequences represented in any of SEQ ID Nos:10-18 or 20, or a homologous sequence thereto. Preferred fragments of the subject *hedgehog* proteins correspond to the N-terminal and C-terminal proteolytic fragments of the mature protein. Bioactive fragments of *hedgehog* polypeptides are described in great detail in PCT publications WO 95/18856 and WO 96/17924.

With respect to bioactive fragments of *hedgehog* polypeptide, preferred *hedgehog* therapeutics include at least 50 (contiguous) amino acid residues of a *hedgehog* polypeptide, more preferably at least 100 (contiguous), and even more preferably at least 150 (contiguous) residues.

Another preferred *hedgehog* polypeptide which can be included in the *hedgehog* therapeutic is an N-terminal fragment of the mature protein having a molecular weight of approximately 19 kDa.

Preferred human *hedgehog* proteins include N-terminal fragments corresponding approximately to residues 24-197 of SEQ ID No. 15, 28-202 of SEQ ID No. 16, and 23-198 of SEQ ID No. 17. By "corresponding approximately" it is meant that the sequence of interest is at most 20 amino acid residues different in length to the reference sequence, though more preferably at most 5, 10 or 15 amino acid different in length.

As described above for recombinant polypeptides, isolated *hedgehog* polypeptides can include all or a portion of the amino acid sequences represented in SEQ ID No:8, SEQ ID No:9, SEQ ID No:10, SEQ ID No:11, SEQ ID No:12, SEQ ID No:13 or SEQ ID No:14, or a homologous sequence thereto. Preferred fragments of the subject *hedgehog* proteins correspond to the N-terminal and C-terminal proteolytic fragments of the mature protein. Bioactive fragments of *hedgehog* polypeptides are described in great detail in PCT publications WO 95/18856 and WO 96/17924.

Still other preferred *hedgehog* polypeptides includes an amino acid sequence represented by the formula A-B wherein: (i) A represents all or the portion of the amino acid sequence designated by residues 1-168 of SEQ ID No:21; and B represents at least one amino acid residue of the amino acid sequence designated by residues 169-221 of SEQ ID No:21; (ii) A represents all or the portion of the amino acid sequence designated by residues 24-193 of SEQ ID No:15;

and B represents at least one amino acid residue of the amino acid sequence designated by residues 194-250 of SEQ ID No:15; (iii) A represents all or the portion of the amino acid sequence designated by residues 25-193 of SEQ ID No:13; and B represents at least one amino acid residue of the amino acid sequence designated by residues 194-250 of SEQ ID No:13; (iv) A represents all or the portion of the amino acid sequence designated by residues 23-193 of SEQ ID No:11; and B represents at least one amino acid residue of the amino acid sequence designated by residues 194-250 of SEQ ID No:11; (v) A represents all or the portion of the amino acid sequence designated by residues 28-197 of SEQ ID No:12; and B represents at least one amino acid residue of the amino acid sequence designated by residues 198-250 of SEQ ID No:12; (vi) A represents all or the portion of the amino acid sequence designated by residues 29-197 of SEQ ID No:16; and B represents at least one amino acid residue of the amino acid sequence designated by residues 198-250 of SEQ ID No:16; or (vii) A represents all or the portion of the amino acid sequence designated by residues 23-193 of SEQ ID No. 17, and B represents at least one amino acid residue of the amino acid sequence designated by residues 194-250 of SEQ ID No. 17. In certain preferred embodiments, A and B together represent a contiguous polypeptide sequence designated sequence, A represents at least 25, 50, 75, 100, 125 or 150 (contiguous) amino acids of the designated sequence, and B represents at least 5, 10, or 20 (contiguous) amino acid residues of the amino acid sequence designated by corresponding entry in the sequence listing, and A and B together preferably represent a contiguous sequence corresponding to the sequence listing entry. Similar fragments from other *hedgehog* also contemplated, e.g., fragments which correspond to the preferred fragments from the sequence listing entries which are enumerated above. In preferred embodiments, the *hedgehog* polypeptide includes a C-terminal glycine (or other appropriate residue) which is derivatized with a cholesterol.

Isolated peptidyl portions of *hedgehog* proteins can be obtained by screening peptides recombinantly produced from the corresponding fragment of the nucleic acid encoding such peptides. In addition, fragments can be chemically synthesized using techniques known in the art such as conventional Merrifield solid phase f-Moc or t-Boc chemistry. For example, a *hedgehog* polypeptide of the present invention may be arbitrarily divided into fragments of desired length with no overlap of the fragments, or preferably divided into overlapping fragments of a desired length. The fragments can be produced (recombinantly or by chemical synthesis) and tested to identify those peptidyl fragments which can function as either agonists or antagonists of a wild-type (e.g., "authentic") *hedgehog* protein. For example, Román et al. (1994) *Eur J Biochem* 222:65-73 describe the use of competitive-binding assays using short, overlapping synthetic peptides from larger proteins to identify binding domains.

The recombinant *hedgehog* polypeptides of the present invention also include homologs of the authentic *hedgehog* proteins, such as versions of those protein which are resistant to proteolytic cleavage, as for example, due to mutations which alter potential cleavage sequences or which inactivate an enzymatic activity associated with the protein. *Hedgehog* homologs of the present invention also include proteins which have been post-translationally modified in a manner different than the authentic protein. Exemplary derivatives of *hedgehog* proteins include polypeptides which lack N-glycosylation sites (e.g. to produce an unglycosylated protein), which lack sites for cholesterolization, and/or which lack N-terminal and/or C-terminal sequences.

Modification of the structure of the subject *hedgehog* polypeptides can also be for such purposes as enhancing therapeutic or prophylactic efficacy, or stability (e.g., *ex vivo* shelf life and resistance to proteolytic degradation *in vivo*). Such modified peptides, when designed to retain at least one activity of the naturally-occurring form of the protein, are considered functional equivalents of the *hedgehog* polypeptides described in more detail herein. Such modified peptides can be produced, for instance, by amino acid substitution, deletion, or addition.

It is well known in the art that one could reasonably expect that certain isolated replacements of amino acids, e.g., replacement of an amino acid residue with another related amino acid (i.e. isosteric and/or isoelectric mutations), can be carried out without major effect on the biological activity of the resulting molecule. Conservative replacements are those that take place within a family of amino acids that are related in their side chains. Genetically encoded amino acids can be divided into four families: (1) acidic = aspartate, glutamate; (2) basic = lysine, arginine, histidine; (3) nonpolar = alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar = glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine. Phenylalanine, tryptophan, and tyrosine are sometimes classified jointly as aromatic amino acids. In similar fashion, the amino acid repertoire can be grouped as (1) acidic = aspartate, glutamate; (2) basic = lysine, arginine histidine, (3) aliphatic = glycine, alanine, valine, leucine, isoleucine, serine, threonine, with serine and threonine optionally be grouped separately as aliphatic-hydroxyl; (4) aromatic = phenylalanine, tyrosine, tryptophan; (5) amide = asparagine, glutamine; and (6) sulfur -containing = cysteine and methionine. (see, for example, *Biochemistry*, 2nd ed., Ed. by L. Stryer, WH Freeman and Co.: 1981). Whether a change in the amino acid sequence of a peptide results in a functional *hedgehog* homolog (e.g. functional in the sense that it acts to mimic or antagonize the wild-type form) can be readily determined by assessing the ability of the variant peptide to produce a response in cells in a fashion similar to the wild-type protein, or competitively inhibit such a response. Polypeptides in which more than one replacement has taken place can readily be tested in the same manner.

It is specifically contemplated that the methods of the present invention can be carried using homologs of naturally occurring hedgehog proteins. In one embodiment, the invention contemplates using hedgehog polypeptides generated by combinatorial mutagenesis. Such methods, as are known in the art, are convenient for generating both point and truncation mutants, and can be especially useful for identifying potential variant sequences (e.g. homologs) that are functional in binding to a receptor for *hedgehog* proteins. The purpose of screening such combinatorial libraries is to generate, for example, novel *hedgehog* homologs which can act as either agonists or antagonist. To illustrate, *hedgehog* homologs can be engineered by the present method to provide more efficient binding to a cognate receptor, such as *patched*, yet still retain at least a portion of an activity associated with *hedgehog*. Thus, combinatorially-derived homologs can be generated to have an increased potency relative to a naturally occurring form of the protein. Likewise, *hedgehog* homologs can be generated by the present combinatorial approach to act as antagonists, in that they are able to mimic, for example, binding to other extracellular matrix components (such as receptors), yet not induce any biological response, thereby inhibiting the action of authentic *hedgehog* or *hedgehog* agonists. Moreover, manipulation of certain domains of *hedgehog* by the present method can provide domains more suitable for use in fusion proteins, such as one that incorporates portions of other proteins which are derived from the extracellular matrix and/or which bind extracellular matrix components.

To further illustrate the state of the art of combinatorial mutagenesis, it is noted that the review article of Gallop et al. (1994) *J Med Chem* 37:1233 describes the general state of the art of combinatorial libraries as of the earlier 1990's. In particular, Gallop et al state at page 1239 "[s]creening the analog libraries aids in determining the minimum size of the active sequence and in identifying those residues critical for binding and intolerant of substitution". In addition, the Ladner et al. PCT publication WO90/02809, the Goeddel et al. U.S. Patent 5,223,408, and the Markland et al. PCT publication WO92/15679 illustrate specific techniques which one skilled in the art could utilize to generate libraries of *hedgehog* variants which can be rapidly screened to identify variants/fragments which retained a particular activity of the *hedgehog* polypeptides. These techniques are exemplary of the art and demonstrate that large libraries of related variants/truncants can be generated and assayed to isolate particular variants without undue experimentation. Gustin et al. (1993) *Virology* 193:653, and Bass et al. (1990) *Proteins: Structure, Function and Genetics* 8:309-314 also describe other exemplary techniques from the art which can be adapted as means for generating mutagenic variants of *hedgehog* polypeptides.

Indeed, it is plain from the combinatorial mutagenesis art that large scale mutagenesis of hedgehog proteins, without any preconceived ideas of which residues were critical to the biological function, and generate wide arrays of variants having equivalent biological activity.

Indeed, it is the ability of combinatorial techniques to screen billions of different variants by high throughput analysis that removes any requirement of *a priori* understanding or knowledge of critical residues.

To illustrate, the amino acid sequences for a population of *hedgehog* homologs or other related proteins are aligned, preferably to promote the highest homology possible. Such a population of variants can include, for example, *hedgehog* homologs from one or more species. Amino acids which appear at each position of the aligned sequences are selected to create a degenerate set of combinatorial sequences. In a preferred embodiment, the variegated library of *hedgehog* variants is generated by combinatorial mutagenesis at the nucleic acid level, and is encoded by a variegated gene library. For instance, a mixture of synthetic oligonucleotides can be enzymatically ligated into gene sequences such that the degenerate set of potential *hedgehog* sequences are expressible as individual polypeptides, or alternatively, as a set of larger fusion proteins (e.g. for phage display) containing the set of *hedgehog* sequences therein.

As illustrated in PCT publication WO 95/18856, to analyze the sequences of a population of variants, the amino acid sequences of interest can be aligned relative to sequence homology. The presence or absence of amino acids from an aligned sequence of a particular variant is relative to a chosen consensus length of a reference sequence, which can be real or artificial.

In an illustrative embodiment, alignment of exons 1, 2 and a portion of exon 3 encoded sequences (e.g. the N-terminal approximately 221 residues of the mature protein) of each of the *Shh* clones produces a degenerate set of *Shh* polypeptides represented by the general formula:

C-G-P-G-R-G-X(1)-G-X(2)-R-R-H-P-K-K-L-T-P-L-A-Y-K-Q-F-I-P-N-V-A-E-
K-T-L-G-A-S-G-R-Y-E-G-K-I-X(3)-R-N-S-E-R-F-K-E-L-T-P-N-Y-N-P-D-I-I-F-
K-D-E-E-N-T-G-A-D-R-L-M-T-Q-R-C-K-D-K-L-N-X(4)-L-A-I-S-V-M-N-X(5)-
W-P-G-V-X(6)-L-R-V-T-E-G-W-D-E-D-G-H-H-X(7)-E-E-S-L-H-Y-E-G-R-A-V-
D-I-T-T-S-D-R-D-X(8)-S-K-Y-G-X(9)-L-X(10)-R-L-A-V-E-A-G-F-D-W-V-Y-
Y-E-S-K-A-H-I-H-C-S-V-K-A-E-N-S-V-A-A-K-S-G-G-C-F-P-G-S-A-X(11)-V-
X(12)-L-X(13)-X(14)-G-G-X(15)-K-X(16)-V-K-D-L-X(17)-P-G-D-X(18)-V-L-
A-A-D-X(19)-X(20)-G-X(21)-L-X(22)-X(23)-S-D-F-X(24)-X(25)-F-X(26)-D-R
(SEQ ID No: 21)

wherein each of the degenerate positions "X" can be an amino acid which occurs in that position in one of the human, mouse, chicken or zebrafish *Shh* clones, or, to expand the library, each X can also be selected from amongst amino acid residue which would be conservative substitutions for the amino acids which appear naturally in each of those positions. For instance, Xaa(1) represents Gly, Ala, Val, Leu, Ile, Phe, Tyr or Trp ; Xaa(2) represents Arg, His or Lys; Xaa(3) represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(4) represents Gly, Ala, Val, Leu, Ile, Ser or

Thr; Xaa(5) represents Lys, Arg, His, Asn or Gln; Xaa(6) represents Lys, Arg or His; Xaa(7) represents Ser, Thr, Tyr, Trp or Phe; Xaa(8) represents Lys, Arg or His; Xaa(9) represents Met, Cys, Ser or Thr; Xaa(10) represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(11) represents Leu, Val, Met, Thr or Ser; Xaa(12) represents His, Phe, Tyr, Ser, Thr, Met or Cys; Xaa(13) represents Gln, Asn, Glu, or Asp; Xaa(14) represents His, Phe, Tyr, Thr, Gln, Asn, Glu or Asp; Xaa(15) represents Gln, Asn, Glu, Asp, Thr, Ser, Met or Cys; Xaa(16) represents Ala, Gly, Cys, Leu, Val or Met; Xaa(17) represents Arg, Lys, Met, Ile, Asn, Asp, Glu, Gln, Ser, Thr or Cys; Xaa(18) represents Arg, Lys, Met or Ile; Xaa(19) represents Ala, Gly, Cys, Asp, Glu, Gln, Asn, Ser, Thr or Met; Xaa(20) represents Ala, Gly, Cys, Asp, Asn, Glu or Gln; Xaa(21) represents Arg, Lys, Met, Ile, Asn, Asp, Glu or Gln; Xaa(22) represent Leu, Val, Met or Ile; Xaa(23) represents Phe, Tyr, Thr, His or Trp; Xaa(24) represents Ile, Val, Leu or Met; Xaa(25) represents Met, Cys, Ile, Leu, Val, Thr or Ser; Xaa(26) represents Leu, Val, Met, Thr or Ser. In an even more expansive library, each X can be selected from any amino acid.

In similar fashion, alignment of each of the human, mouse, chicken and zebrafish *hedgehog* clones, can provide a degenerate polypeptide sequence represented by the general formula:

C-G-P-G-R-G-X(1)-X(2)-X(3)-R-R-X(4)-X(5)-X(6)-P-K-X(7)-L-X(8)-P-L-X(9)-Y-K-Q-F-X(10)-P-X(11)-X(12)-X(13)-E-X(14)-T-L-G-A-S-G-X(15)-X(16)-E-G-X(17)-X(18)-X(19)-R-X(20)-S-E-R-F-X(21)-X(22)-L-T-P-N-Y-N-P-D-I-I-F-K-D-E-E-N-X(23)-G-A-D-R-L-M-T-X(24)-R-C-K-X(25)-X(26)-X(27)-N-X(28)-L-A-I-S-V-M-N-X(29)-W-P-G-V-X(30)-L-R-V-T-E-G-X(31)-D-E-D-G-H-H-X(32)-X(33)-X(34)-S-L-H-Y-E-G-R-A-X(35)-D-I-T-T-S-D-R-D-X(36)-X(37)-K-Y-G-X(38)-L-X(39)-R-L-A-V-E-A-G-F-D-W-V-Y-Y-E-S-X(40)-X(41)-H-X(42)-H-X(43)-S-V-K-X(44)-X(45) (SEQ IDNo:22)

wherein, as above, each of the degenerate positions "X" can be an amino acid which occurs in a corresponding position in one of the wild-type clones, and may also include amino acid residue which would be conservative substitutions, or each X can be any amino acid residue. In an exemplary embodiment, Xaa(1) represents Gly, Ala, Val, Leu, Ile, Pro, Phe or Tyr; Xaa(2) represents Gly, Ala, Val, Leu or Ile; Xaa(3) represents Gly, Ala, Val, Leu, Ile, Lys, His or Arg; Xaa(4) represents Lys, Arg or His; Xaa(5) represents Phe, Trp, Tyr or an amino acid gap; Xaa(6) represents Gly, Ala, Val, Leu, Ile or an amino acid gap; Xaa(7) represents Asn, Gln, His, Arg or Lys; Xaa(8) represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(9) represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(10) represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(11) represents Ser, Thr, Gln or Asn; Xaa(12) represents Met, Cys, Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(13) represents Gly, Ala, Val, Leu, Ile or Pro; Xaa(14) represents Arg, His or Lys; Xaa(15) represents Gly, Ala, Val, Leu, Ile, Pro, Arg, His or Lys; Xaa(16) represents Gly, Ala, Val, Leu, Ile, Phe or

Tyr; Xaa(17) represents Arg, His or Lys; Xaa(18) represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(19) represents Thr or Ser; Xaa(20) represents Gly, Ala, Val, Leu, Ile, Asn or Gln; Xaa(21) represents Arg, His or Lys; Xaa(22) represents Asp or Glu; Xaa(23) represents Ser or Thr; Xaa(24) represents Glu, Asp, Gln or Asn; Xaa(25) represents Glu or Asp; Xaa(26) represents Arg, His or Lys; Xaa(27) represents Gly, Ala, Val, Leu or Ile; Xaa(28) represents Gly, Ala, Val, Leu, Ile, Thr or Ser; Xaa(29) represents Met, Cys, Gln, Asn, Arg, Lys or His; Xaa(30) represents Arg, His or Lys; Xaa(31) represents Trp, Phe, Tyr, Arg, His or Lys; Xaa(32) represents Gly, Ala, Val, Leu, Ile, Ser, Thr, Tyr or Phe; Xaa(33) represents Gln, Asn, Asp or Glu; Xaa(34) represents Asp or Glu; Xaa(35) represents Gly, Ala, Val, Leu, or Ile; Xaa(36) represents Arg, His or Lys; Xaa(37) represents Asn, Gln, Thr or Ser; Xaa(38) represents Gly, Ala, Val, Leu, Ile, Ser, Thr, Met or Cys; Xaa(39) represents Gly, Ala, Val, Leu, Ile, Thr or Ser; Xaa(40) represents Arg, His or Lys; Xaa(41) represents Asn, Gln, Gly, Ala, Val, Leu or Ile; Xaa(42) represents Gly, Ala, Val, Leu or Ile; Xaa(43) represents Gly, Ala, Val, Leu, Ile, Ser, Thr or Cys; Xaa(44) represents Gly, Ala, Val, Leu, Ile, Thr or Ser; and Xaa(45) represents Asp or Glu.

There are many ways by which the library of potential *hedgehog* homologs can be generated from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence can be carried out in an automatic DNA synthesizer, and the synthetic genes then ligated into an appropriate expression vector. The purpose of a degenerate set of genes is to provide, in one mixture, all of the sequences encoding the desired set of potential *hedgehog* sequences. The synthesis of degenerate oligonucleotides is well known in the art (see for example, Narang, SA (1983) *Tetrahedron* 39:3; Itakura et al. (1981) *Recombinant DNA, Proc 3rd Cleveland Sympos. Macromolecules*, ed. AG Walton, Amsterdam: Elsevier pp273-289; Itakura et al. (1984) *Annu. Rev. Biochem.* 53:323; Itakura et al. (1984) *Science* 198:1056; Ike et al. (1983) *Nucleic Acid Res.* 11:477. Such techniques have been employed in the directed evolution of other proteins (see, for example, Scott et al. (1990) *Science* 249:386-390; Roberts et al. (1992) *PNAS* 89:2429-2433; Devlin et al. (1990) *Science* 249: 404-406; Cwirla et al. (1990) *PNAS* 87: 6378-6382; as well as U.S. Patents Nos. 5,223,409, 5,198,346, and 5,096,815).

A wide range of techniques are known in the art for screening gene products of combinatorial libraries made by point mutations, and for screening cDNA libraries for gene products having a certain property. Such techniques will be generally adaptable for rapid screening of the gene libraries generated by the combinatorial mutagenesis of *hedgehog* homologs. The most widely used techniques for screening large gene libraries typically comprises cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates relatively easy isolation of the

vector encoding the gene whose product was detected. Each of the illustrative assays described below are amenable to high through-put analysis as necessary to screen large numbers of degenerate *hedgehog* sequences created by combinatorial mutagenesis techniques.

In one embodiment, the combinatorial library is designed to be secreted (e.g. the polypeptides of the library all include a signal sequence but no transmembrane or cytoplasmic domains), and is used to transfect a eukaryotic cell that can be co-cultured with peripheal nerve cells. A functional *hedgehog* protein secreted by the cells expressing the combinatorial library will diffuse to neighboring T lymphocytes and induce a particular biological response, such as proliferation or differentiation. The pattern of detection of such a change in phenotype will resemble a gradient function, and will allow the isolation (generally after several repetitive rounds of selection) of cells producing *hedgehog* homologs active as neurotrophic agents. Likewise, *hedgehog* antagonists can be selected in similar fashion by the ability of the cell producing a functional antagonist to protect neighboring cells (e.g., to inhibit proliferation) from the effect of wild-type *hedgehog* added to the culture media.

To illustrate, target T lymphocytes are cultured in 24-well microtitre plates. Other eukaryotic cells are transfected with the combinatorial *hedgehog* gene library and cultured in cell culture inserts (e.g. Collaborative Biomedical Products, Catalog #40446) that are able to fit into the wells of the microtitre plate. The cell culture inserts are placed in the wells such that recombinant *hedgehog* homologs secreted by the cells in the insert can diffuse through the porous bottom of the insert and contact the target cells in the microtitre plate wells. After a period of time sufficient for functional forms of a *hedgehog* protein to produce a measurable response in the target cells, such as growth state, the inserts are removed and the effect of the variant *hedgehog* proteins on the target cells determined. Cells from the inserts corresponding to wells which score positive for activity can be split and re-cultured on several inserts, the process being repeated until the active clones are identified.

In yet another screening assay, the candidate *hedgehog* gene products are displayed on the surface of a cell or viral particle, and the ability of particular cells or viral particles to associate with a *hedgehog*-binding moiety (such as the *patched* protein or other *hedgehog* receptor) via this gene product is detected in a "panning assay". Such panning steps can be carried out on cells cultured from embryos. For instance, the gene library can be cloned into the gene for a surface membrane protein of a bacterial cell, and the resulting fusion protein detected by panning (Ladner et al., WO 88/06630; Fuchs et al. (1991) *Bio/Technology* 9:1370-1371; and Goward et al. (1992) *TIBS* 18:136-140). In a similar fashion, fluorescently labeled molecules which bind *hedgehog* can be used to score for potentially functional *hedgehog* homologs. Cells can be

visually inspected and separated under a fluorescence microscope, or, where the morphology of the cell permits, separated by a fluorescence-activated cell sorter.

In an alternate embodiment, the gene library is expressed as a fusion protein on the surface of a viral particle. For instance, in the filamentous phage system, foreign peptide sequences can be expressed on the surface of infectious phage, thereby conferring two significant benefits. First, since these phage can be applied to affinity matrices at very high concentrations, large number of phage can be screened at one time. Second, since each infectious phage displays the combinatorial gene product on its surface, if a particular phage is recovered from an affinity matrix in low yield, the phage can be amplified by another round of infection. The group of almost identical *E.coli* filamentous phages M13, fd, and f1 are most often used in phage display libraries, as either of the phage gIII or gVIII coat proteins can be used to generate fusion proteins without disrupting the ultimate packaging of the viral particle (Ladner et al. PCT publication WO 90/02909; Garrard et al., PCT publication WO 92/09690; Marks et al. (1992) J. Biol. Chem. 267:16007-16010; Griffiths et al. (1993) EMBO J 12:725-734; Clackson et al. (1991) Nature 352:624-628; and Barbas et al. (1992) PNAS 89:4457-4461).

In an illustrative embodiment, the recombinant phage antibody system (RPAS, Pharmacia Catalog number 27-9400-01) can be easily modified for use in expressing and screening *hedgehog* combinatorial libraries. For instance, the pCANTAB 5 phagemid of the RPAS kit contains the gene which encodes the phage gIII coat protein. The *hedgehog* combinatorial gene library can be cloned into the phagemid adjacent to the gIII signal sequence such that it will be expressed as a gIII fusion protein. After ligation, the phagemid is used to transform competent *E. coli* TG1 cells. Transformed cells are subsequently infected with M13KO7 helper phage to rescue the phagemid and its candidate *hedgehog* gene insert. The resulting recombinant phage contain phagemid DNA encoding a specific candidate *hedgehog*, and display one or more copies of the corresponding fusion coat protein. The phage-displayed candidate *hedgehog* proteins which are capable of binding an *hedgehog* receptor are selected or enriched by panning. For instance, the phage library can be applied to cells which express the *patched* protein and unbound phage washed away from the cells. The bound phage is then isolated, and if the recombinant phage express at least one copy of the wild type gIII coat protein, they will retain their ability to infect *E. coli*. Thus, successive rounds of reinfection of *E. coli*, and panning will greatly enrich for *hedgehog* homologs, which can then be screened for further biological activities in order to differentiate agonists and antagonists.

Combinatorial mutagenesis has a potential to generate very large libraries of mutant proteins, e.g., in the order of 10^{26} molecules. Combinatorial libraries of this size may be

technically challenging to screen even with high throughput screening assays such as phage display. To overcome this problem, a new technique has been developed recently, recursive ensemble mutagenesis (REM), which allows one to avoid the very high proportion of non-functional proteins in a random library and simply enhances the frequency of functional proteins, thus decreasing the complexity required to achieve a useful sampling of sequence space. REM is an algorithm which enhances the frequency of functional mutants in a library when an appropriate selection or screening method is employed (Arkin and Yourvan, 1992, *PNAS USA* 89:7811-7815; Yourvan et al., 1992, *Parallel Problem Solving from Nature*, 2., In Maenner and Manderick, eds., Elsevir Publishing Co., Amsterdam, pp. 401-410; Delgrave et al., 1993, *Protein Engineering* 6(3):327-331).

The invention also provides for reduction of the *hedgehog* protein to generate mimetics, e.g. peptide or non-peptide agents, which are able to disrupt binding of a *hedgehog* polypeptide of the present invention with an *hedgehog* receptor. Thus, such mutagenic techniques as described above are also useful to map the determinants of the *hedgehog* proteins which participate in protein-protein interactions involved in, for example, binding of the subject *hedgehog* polypeptide to other extracellular matrix components. To illustrate, the critical residues of a subject *hedgehog* polypeptide which are involved in molecular recognition of an *hedgehog* receptor such as *patched* can be determined and used to generate *hedgehog*-derived peptidomimetics which competitively inhibit binding of the authentic *hedgehog* protein with that moiety. By employing, for example, scanning mutagenesis to map the amino acid residues of each of the subject *hedgehog* proteins which are involved in binding other extracellular proteins, peptidomimetic compounds can be generated which mimic those residues of the *hedgehog* protein which facilitate the interaction. Such mimetics may then be used to interfere with the normal function of a *hedgehog* protein. For instance, non-hydrolyzable peptide analogs of such residues can be generated using benzodiazepine (e.g., see Freidinger et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), azepine (e.g., see Huffman et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), substituted gama lactam rings (Garvey et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), keto-methylene pseudopeptides (Ewenson et al. (1986) *J Med Chem* 29:295; and Ewenson et al. in *Peptides: Structure and Function* (Proceedings of the 9th American Peptide Symposium) Pierce Chemical Co. Rockland, IL, 1985), β -turn dipeptide cores (Nagai et al. (1985) *Tetrahedron Lett* 26:647; and Sato et al. (1986) *J Chem Soc Perkin Trans* 1:1231), and β -aminoalcohols (Gordon et al. (1985) *Biochem Biophys Res Commun* 126:419; and Dann et al. (1986) *Biochem Biophys Res Commun* 134:71).

Recombinantly produced forms of the hedgehog proteins can be produced using, e.g., expression vectors containing a nucleic acid encoding a *hedgehog* polypeptide, operably linked to at least one transcriptional regulatory sequence. Operably linked is intended to mean that the nucleotide sequence is linked to a regulatory sequence in a manner which allows expression of the nucleotide sequence. Regulatory sequences are art-recognized and are selected to direct expression of a *hedgehog* polypeptide. Accordingly, the term transcriptional regulatory sequence includes promoters, enhancers and other expression control elements. Such regulatory sequences are described in Goeddel; *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). For instance, any of a wide variety of expression control sequences, sequences that control the expression of a DNA sequence when operatively linked to it, may be used in these vectors to express DNA sequences encoding *hedgehog* polypeptide. Such useful expression control sequences, include, for example, a viral LTR, such as the LTR of the Moloney murine leukemia virus, the early and late promoters of SV40, adenovirus or cytomegalovirus immediate early promoter, the lac system, the trp system, the TAC or TRC system, T7 promoter whose expression is directed by T7 RNA polymerase, the major operator and promoter regions of phage λ , the control regions for fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase, e.g., Pho5, the promoters of the yeast α -mating factors, the polyhedron promoter of the baculovirus system and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof. It should be understood that the design of the expression vector may depend on such factors as the choice of the host cell to be transformed and/or the type of protein desired to be expressed. Moreover, the vector's copy number, the ability to control that copy number and the expression of any other proteins encoded by the vector, such as antibiotic markers, should also be considered.

In addition to providing a ready source of hedgehog polypeptides for purification, the gene constructs of the present invention can also be used as a part of a gene therapy protocol to deliver nucleic acids encoding either an agonistic or antagonistic form of a *hedgehog* polypeptide. Thus, another aspect of the invention features expression vectors for *in vivo* transfection of a *hedgehog* polypeptide in particular cell types so as cause ectopic expression of a *hedgehog* polypeptide in an T lymphocytes or other cells associated therewith.

Formulations of such expression constructs may be administered in any biologically effective carrier, e.g. any formulation or composition capable of effectively delivering the recombinant gene to cells *in vivo*. Approaches include insertion of the hedgehog coding sequence in viral vectors including recombinant retroviruses, adenovirus, adeno-associated virus, and herpes simplex virus-1, or recombinant bacterial or eukaryotic plasmids. Viral vectors transfect

cells directly; plasmid DNA can be delivered with the help of, for example, cationic liposomes (lipofectin) or derivatized (e.g. antibody conjugated), polylysine conjugates, gramicidin S, artificial viral envelopes or other such intracellular carriers, as well as direct injection of the gene construct or CaPO_4 precipitation carried out *in vivo*. It will be appreciated that because

transduction of appropriate target cells represents the critical first step in gene therapy, choice of the particular gene delivery system will depend on such factors as the phenotype of the intended target and the route of administration, e.g. locally or systemically. Furthermore, it will be recognized that the particular gene construct provided for *in vivo* transduction of *hedgehog* expression are also useful for *in vitro* transduction of cells, such as for use in the *ex vivo* tissue culture systems described below.

A preferred approach for *in vivo* introduction of nucleic acid into a cell is by use of a viral vector containing nucleic acid, e.g. a cDNA, encoding the particular form of the *hedgehog* polypeptide desired. Infection of cells with a viral vector has the advantage that a large proportion of the targeted cells can receive the nucleic acid. Additionally, molecules encoded within the viral vector, e.g., by a cDNA contained in the viral vector, are expressed efficiently in cells which have taken up viral vector nucleic acid.

Retrovirus vectors and adeno-associated virus vectors are generally understood to be the recombinant gene delivery system of choice for the transfer of exogenous genes *in vivo*, particularly into humans. These vectors provide efficient delivery of genes into cells, and the transferred nucleic acids are stably integrated into the chromosomal DNA of the host. A major prerequisite for the use of retroviruses is to ensure the safety of their use, particularly with regard to the possibility of the spread of wild-type virus in the cell population. The development of specialized cell lines (termed "packaging cells") which produce only replication-defective retroviruses has increased the utility of retroviruses for gene therapy, and defective retroviruses are well characterized for use in gene transfer for gene therapy purposes (for a review see Miller, A.D. (1990) *Blood* 76:271). Thus, recombinant retrovirus can be constructed in which part of the retroviral coding sequence (*gag*, *pol*, *env*) has been replaced by nucleic acid encoding a *hedgehog* polypeptide and renders the retrovirus replication defective. The replication defective retrovirus is then packaged into virions which can be used to infect a target cell through the use of a helper virus by standard techniques. Protocols for producing recombinant retroviruses and for infecting cells *in vitro* or *in vivo* with such viruses can be found in Current Protocols in Molecular Biology, Ausubel, F.M. et al. (eds.) Greene Publishing Associates, (1989), Sections 9.10-9.14 and other standard laboratory manuals. Examples of suitable retroviruses include pLJ, pZIP, pWE and pEM which are well known to those skilled in the art. Examples of suitable packaging virus lines for preparing both ecotropic and amphotropic retroviral systems include

Crip, Cre, 2 and Am. Retroviruses have been used to introduce a variety of genes into many different cell types, including T lymphocytes, *in vitro* and/or *in vivo* (see for example Eglitis, et al. (1985) *Science* 230:1395-1398; Danos and Mulligan (1988) *Proc. Natl. Acad. Sci. USA* 85:6460-6464; Wilson et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:3014-3018; Armentano et al. (1990) *Proc. Natl. Acad. Sci. USA* 87:6141-6145; Huber et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:8039-8043; Ferry et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:8377-8381; Chowdhury et al. (1991) *Science* 254:1802-1805; van Beusechem et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:7640-7644; Kay et al. (1992) *Human Gene Therapy* 3:641-647; Dai et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:10892-10895; Hwu et al. (1993) *J. Immunol.* 150:4104-4115; U.S. Patent No. 4,868,116; U.S. Patent No. 4,980,286; PCT Application WO 89/07136; PCT Application WO 89/02468; PCT Application WO 89/05345; and PCT Application WO 92/07573).

Furthermore, it has been shown that it is possible to limit the infection spectrum of retroviruses and consequently of retroviral-based vectors, by modifying the viral packaging proteins on the surface of the viral particle (see, for example PCT publications WO93/25234 and WO94/06920). For instance, strategies for the modification of the infection spectrum of retroviral vectors include: coupling antibodies specific for cell surface antigens to the viral *env* protein (Roux et al. (1989) *PNAS* 86:9079-9083; Julan et al. (1992) *J. Gen Virol* 73:3251-3255; and Goud et al. (1983) *Virology* 163:251-254); or coupling cell surface receptor ligands to the viral *env* proteins (Neda et al. (1991) *J Biol Chem* 266:14143-14146). Coupling can be in the form of the chemical cross-linking with a protein or other variety (e.g. lactose to convert the *env* protein to an asialoglycoprotein), as well as by generating fusion proteins (e.g. single-chain antibody/*env* fusion proteins). This technique, while useful to limit or otherwise direct the infection to certain tissue types, can also be used to convert an ecotropic vector in to an amphotropic vector.

Moreover, use of retroviral gene delivery can be further enhanced by the use of tissue- or cell-specific transcriptional regulatory sequences which control expression of the *hedgehog* gene of the retroviral vector.

Another viral gene delivery system useful in the present method utilizes adenovirus-derived vectors. The genome of an adenovirus can be manipulated such that it encodes and expresses a gene product of interest but is inactivated in terms of its ability to replicate in a normal lytic viral life cycle. See for example Berkner et al. (1988) *BioTechniques* 6:616; Rosenfeld et al. (1991) *Science* 252:431-434; and Rosenfeld et al. (1992) *Cell* 68:143-155. Suitable adenoviral vectors derived from the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (e.g., Ad2, Ad3, Ad7 etc.) are well known to those skilled in the art. Recombinant

adenoviruses can be advantageous in certain circumstances in that they can be used to infect a wide variety of cell types, including T lymphocytes. Furthermore, the virus particle is relatively stable and amenable to purification and concentration, and as above, can be modified so as to affect the spectrum of infectivity. Additionally, introduced adenoviral DNA (and foreign DNA contained therein) is not integrated into the genome of a host cell but remains episomal, thereby avoiding potential problems that can occur as a result of insertional mutagenesis in situations where introduced DNA becomes integrated into the host genome (e.g., retroviral DNA). Moreover, the carrying capacity of the adenoviral genome for foreign DNA is large (up to 8 kilobases) relative to other gene delivery vectors (Berkner et al. cited *supra*; Haj-Ahmand and Graham (1986) *J. Virol.* 57:267). Most replication-defective adenoviral vectors currently in use and therefore favored by the present invention are deleted for all or parts of the viral E1 and E3 genes but retain as much as 80% of the adenoviral genetic material (see, e.g., Jones et al. (1979) *Cell* 16:683; Berkner et al., *supra*; and Graham et al. in Methods in Molecular Biology, E.J. Murray, Ed. (Humana, Clifton, NJ, 1991) vol. 7. pp. 109-127). Expression of the inserted *hedgehog* gene can be under control of, for example, the E1A promoter, the major late promoter (MLP) and associated leader sequences, the E3 promoter, or exogenously added promoter sequences.

In addition to viral transfer methods, such as those illustrated above, non-viral methods can also be employed to cause expression of a *hedgehog* polypeptide in the tissue of an animal. Most nonviral methods of gene transfer rely on normal mechanisms used by mammalian cells for the uptake and intracellular transport of macromolecules. In preferred embodiments, non-viral gene delivery systems of the present invention rely on endocytic pathways for the uptake of the *hedgehog* polypeptide gene by the targeted cell. Exemplary gene delivery systems of this type include liposomal derived systems, poly-lysine conjugates, and artificial viral envelopes.

In clinical settings, the gene delivery systems for the therapeutic *hedgehog* gene can be introduced into a patient by any of a number of methods, each of which is familiar in the art. For instance, a pharmaceutical preparation of the gene delivery system can be introduced systemically, e.g. by intravenous injection, and specific transduction of the protein in the target cells occurs predominantly from specificity of transfection provided by the gene delivery vehicle, cell-type or tissue-type expression due to the transcriptional regulatory sequences controlling expression of the receptor gene, or a combination thereof. In other embodiments, initial delivery of the recombinant gene is more limited with introduction into the animal being quite localized. For example, the gene delivery vehicle can be introduced by catheter (see U.S. Patent 5,328,470) or by stereotactic injection (e.g. Chen et al. (1994) *PNAS* 91: 3054-3057). A *hedgehog* expression construct can be delivered in a gene therapy construct to dermal cells by, e.g.,

electroporation using techniques described, for example, by Dev et al. ((1994) *Cancer Treat Rev* 20:105-115).

The pharmaceutical preparation of the gene therapy construct can consist essentially of the gene delivery system in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery system can be produced intact from recombinant cells, e.g. retroviral vectors, the pharmaceutical preparation can comprise one or more cells which produce the gene delivery system.

In yet another embodiment, the hedgehog or ptc therapeutic can be a "gene activation" construct which, by homologous recombination with a genomic DNA, alters the transcriptional regulatory sequences of an endogenous gene. For instance, the gene activation construct can replace the endogenous promoter of a *hedgehog* gene with a heterologous promoter, e.g., one which causes constitutive expression of the *hedgehog* gene or which causes inducible expression of the gene under conditions different from the normal expression pattern of the gene. Other genes in the *patched* signaling pathway can be similarly targeted. A variety of different formats for the gene activation constructs are available. See, for example, the Transkaryotic Therapies, Inc PCT publications WO93/09222, WO95/31560, WO96/29411, WO95/31560 and WO94/12650.

In preferred embodiments, the nucleotide sequence used as the gene activation construct can be comprised of (1) DNA from some portion of the endogenous *hedgehog* gene (exon sequence, intron sequence, promoter sequences, etc.) which direct recombination and (2) heterologous transcriptional regulatory sequence(s) which is to be operably linked to the coding sequence for the genomic *hedgehog* gene upon recombination of the gene activation construct. For use in generating cultures of *hedgehog* producing cells, the construct may further include a reporter gene to detect the presence of the knockout construct in the cell.

The gene activation construct is inserted into a cell, and integrates with the genomic DNA of the cell in such a position so as to provide the heterologous regulatory sequences in operative association with the native *hedgehog* gene. Such insertion occurs by homologous recombination, i.e., recombination regions of the activation construct that are homologous to the endogenous *hedgehog* gene sequence hybridize to the genomic DNA and recombine with the genomic sequences so that the construct is incorporated into the corresponding position of the genomic DNA.

The terms "recombination region" or "targeting sequence" refer to a segment (i.e., a portion) of a gene activation construct having a sequence that is substantially identical to or substantially complementary to a genomic gene sequence, e.g., including 5' flanking sequences

of the genomic gene, and can facilitate homologous recombination between the genomic sequence and the targeting transgene construct.

As used herein, the term "replacement region" refers to a portion of a activation construct which becomes integrated into an endogenous chromosomal location following homologous recombination between a recombination region and a genomic sequence.

The heterologous regulatory sequences, e.g., which are provided in the replacement region, can include one or more of a variety elements, including: promoters (such as constitutive or inducible promoters), enhancers, negative regulatory elements, locus control regions, transcription factor binding sites, or combinations thereof. Promoters/enhancers which may be used to control the expression of the targeted gene *in vivo* include, but are not limited to, the cytomegalovirus (CMV) promoter/enhancer (Karasuyama et al., 1989, *J. Exp. Med.*, 169:13), the human β -actin promoter (Gunning et al. (1987) *PNAS* 84:4831-4835), the glucocorticoid-inducible promoter present in the mouse mammary tumor virus long terminal repeat (MMTV LTR) (Klessig et al. (1984) *Mol. Cell Biol.* 4:1354-1362), the long terminal repeat sequences of Moloney murine leukemia virus (MuLV LTR) (Weiss et al. (1985) *RNA Tumor Viruses*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York), the SV40 early or late region promoter (Bernoist et al. (1981) *Nature* 290:304-310; Templeton et al. (1984) *Mol. Cell Biol.*, 4:817; and Sprague et al. (1983) *J. Virol.*, 45:773), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (RSV) (Yamamoto et al., 1980, *Cell*, 22:787-797), the herpes simplex virus (HSV) thymidine kinase promoter/enhancer (Wagner et al. (1981) *PNAS* 82:3567-71), and the herpes simplex virus LAT promoter (Wolfe et al. (1992) *Nature Genetics*, 1:379-384).

In an exemplary embodiment, portions of the 5' flanking region of the human Shh gene are amplified using primers which add restriction sites, to generate the following fragments

5' -gcgcgccttcgaaGCGAGGCAGCCAGCGAGGGAGAGAGCGAGCGGGCGAGCCGGAGC-GAGGAAatcgatgcgcgc (primer 1)

5' -gcgcgcagatctGCGAAAGCGCAAGAGAGAGCGCACACGCACACACCCGCCGCGCG-CACTCGggatccgcgcgc (primer 2)

As illustrated, primer 1 includes a 5' non-coding region of the human Shh gene and is flanked by an *AsuII* and *Clal* restriction sites. Primer 2 includes a portion of the 5' non-coding region

immediately 3' to that present in primer 1. The hedgehog gene sequence is flanked by XhoII and BamHI restriction sites. The purified amplimers are cut with each of the enzymes as appropriate.

The vector pCDNA1.1 (Invitrogen) includes a CMV promoter. The plasmid is cut with with AsuII, which cleaves just 3' to the CMV promoter sequence. The AsuII/ClaI fragment of primer 1 is ligated to the AsuII cleavage site of the pcDNA vector. The ClaI/AsuII ligation destroys the AsuII site at the 3' end of a properly inserted primer 1.

The vector is then cut with BamHI, and an XhoII/BamHI fragment of primer 2 is ligated to the BamHI cleavage site. As above, the BamHI/XhoII ligation destroys the BamHI site at the 5' end of a properly inserted primer 2.

Individual colonies are selected, cut with AsuII and BamHI, and the size of the AsuII/BamHI fragment determined. Colonies in which both the primer 1 and primer 2 sequences are correctly inserted are further amplified, an cut with AsuII and BamHI to produce the gene activation construct

cgaagcgagggcagccagcgaagggagagagcgaagcggcgagccggagcgaggaaATCGAAGGTTTC
GAATCCTTCCCCACCATCACTTTCAAAGTCCGAAAGAATCTGCTCCCTGCTTGTGTGTTG
GAGGTCGCTGAGTAGTGCGCGAGTAAAATTTAAGCTACAACAAGGCAAGGCTTGACCGACAATTG
CATGAAGAATCTGCTTAGGGTTAGGCGTTTTGCGCTGCTTCGCGATGTACGGGCCAGATATACGC
GTTGACATTGATTATTGACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCA
TATATGGAGTTCCGCGTTACATACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCC
CCGCCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGAC
GTCAATGGGTGGACTATTTACGGTAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCCA
AGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACATGAC
CTTATGGGACTTTCTACTTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGTATGC
GGTTTTTGGCAGTACATCAATGGCGTGGATAGCGGTTTGAATCACGGGGATTTCGAAGTCTCCAC
CCCATTGACGTCAATGGGAGTTTGTTTTGGCACCAAAATCAACGGGACTTTCCAAAATGTCTGTAA
CAACTCCGCCCCATTGACGCAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAG
CTCTCTGGCTAACTAGAGAACCCTGCTTACTGGCTTATCGAAATTAATACGACTCACTATAGG
GAGACCCAAGCTTGGTACCGAGCTCGGATCgatctgggaaagcgcaagagagagcgcacacgcac
acacccgcccgcgcgcactcgg

In this construct, the flanking primer 1 and primer 2 sequences provide the recombination region which permits the insertion of the CMV promoter in front of the coding sequence for the human *Shh* gene. Other heterologous promoters (or other transcriptional regulatory sequences) can be inserted in a genomic *hedgehog* gene by a similar method.

In still other embodiments, the replacement region merely deletes a negative transcriptional control element of the native gene, e.g., to activate expression, or ablates a positive control element, e.g., to inhibit expression of the targeted gene.

5 *V. Exemplary ptc therapeutic compounds.*

In another embodiment, the subject method is carried out using a ptc therapeutic composition. Such compositions can be generated with, for example, compounds which bind to patched and alter its signal transduction activity, compounds which alter the binding and/or enzymatic activity of a protein (e.g., intracellular) involved in patched signal pathway, and
10 compounds which alter the level of expression of a hedgehog protein, a patched protein or a protein involved in the intracellular signal transduction pathway of patched.

The availability of purified and recombinant *hedgehog* polypeptides facilitates the generation of assay systems which can be used to screen for drugs, such as small organic molecules, which are either agonists or antagonists of the normal cellular function of a *hedgehog* and/or patched protein, particularly their role in the pathogenesis of peripheral nerve proliferation and/or differentiation. In one embodiment, the assay evaluates the ability of a compound to modulate binding between a *hedgehog* polypeptide and a *hedgehog* receptor such as *patched*. In other embodiments, the assay merely scores for the ability of a test compound to alter the signal transduction activity of the *patched* protein. In this manner, a variety of *hedgehog* and/or *ptc*
15 therapeutics, both agonists (inhibit T-cell maturation) and antagonist (promote T-cell maturation) can be identified. A variety of assay formats will suffice and, in light of the present disclosure, will be comprehended by skilled artisan.

In many drug screening programs which test libraries of compounds and natural extracts, high throughput assays are desirable in order to maximize the number of compounds surveyed in
25 a given period of time. Assays which are performed in cell-free systems, such as may be derived with purified or semi-purified proteins, are often preferred as "primary" screens in that they can be generated to permit rapid development and relatively easy detection of an alteration in a molecular target which is mediated by a test compound. Moreover, the effects of cellular toxicity and/or bioavailability of the test compound can be generally ignored in the *in vitro*
30 system, the assay instead being focused primarily on the effect of the drug on the molecular target as may be manifest in an alteration of binding affinity with receptor proteins.

Accordingly, in an exemplary screening assay for *ptc* therapeutics, the compound of interest is contacted with a mixture including a *hedgehog* receptor protein (e.g., a cell expressing

the *patched* receptor) and a hedgehog protein under conditions in which it is ordinarily capable of binding the *hedgehog* protein. To the mixture is then added a composition containing a test compound. Detection and quantification of receptor/*hedgehog* complexes provides a means for determining the test compound's efficacy at inhibiting (or potentiating) complex formation between the receptor protein and the *hedgehog* polypeptide. The efficacy of the compound can be assessed by generating dose response curves from data obtained using various concentrations of the test compound. Moreover, a control assay can also be performed to provide a baseline for comparison. In the control assay, isolated and purified *hedgehog* polypeptide is added to the receptor protein, and the formation of receptor/*hedgehog* complex is quantitated in the absence of the test compound.

In other embodiments, a ptc therapeutic of the present invention is one which disrupts the association of *patched* with *smoothened*.

Agonist and antagonists of peripheral nerve maintenance can be distinguished, and the efficacy of the compound can be assessed, by subsequent testing with T lymphocytes, e.g., in culture.

In an illustrative embodiment, the polypeptide utilized as a *hedgehog* receptor can be generated from the *patched* protein. Accordingly, an exemplary screening assay includes all or a suitable portion of the *patched* protein which can be obtained from, for example, the human *patched* gene (GenBank U43148) or other vertebrate sources (see GenBank Accession numbers U40074 for chicken *patched* and U46155 for mouse *patched*), as well as from drosophila (GenBank Accession number M28999) or other invertebrate sources. The *patched* protein can be provided in the screening assay as a whole protein (preferably expressed on the surface of a cell), or alternatively as a fragment of the full length protein which binds to *hedgehog* polypeptides, e.g., as one or both of the substantial extracellular domains (e.g. corresponding to residues Asn120-Ser438 and/or Arg770-Trp1027 of the human *patched* protein - which are also potential antagonists of *hedgehog*-dependent signal transduction). For instance, the *patched* protein can be provided in soluble form, as for example a preparation of one of the extracellular domains, or a preparation of both of the extracellular domains which are covalently connected by an unstructured linker (see, for example, Huston et al. (1988) PNAS 85:4879; and U.S. Patent No. 5,091,513). In other embodiments, the protein can be provided as part of a liposomal preparation or expressed on the surface of a cell. The *patched* protein can be derived from a recombinant gene, e.g., being ectopically expressed in a heterologous cell. For instance, the protein can be expressed on oocytes, mammalian cells (e.g., COS, CHO, 3T3 or the like), or yeast cell by standard recombinant DNA techniques. These recombinant cells can be used for

receptor binding, signal transduction or gene expression assays. Marigo et al. (1996) *Development* 122:1225-1233 illustrates a binding assay of human *hedgehog* to chick *patched* protein ectopically expressed in *Xenopus laevis* oocytes. The assay system of Marigo et al. can be adapted to the present drug screening assays. As illustrated in that reference, *Shh* binds to the *patched* protein in a selective, saturable, dose-dependent manner, thus demonstrating that *patched* is a receptor for *Shh*.

Complex formation between the *hedgehog* polypeptide and a *hedgehog* receptor may be detected by a variety of techniques. For instance, modulation of the formation of complexes can be quantitated using, for example, detectably labelled proteins such as radiolabelled, fluorescently labelled, or enzymatically labelled *hedgehog* polypeptides, by immunoassay, or by chromatographic detection.

Typically, for cell-free assays, it will be desirable to immobilize either the *hedgehog* receptor or the *hedgehog* polypeptide to facilitate separation of receptor/*hedgehog* complexes from uncomplexed forms of one of the proteins, as well as to accommodate automation of the assay. In one embodiment, a fusion protein can be provided which adds a domain that allows the protein to be bound to a matrix. For example, glutathione-S-transferase/receptor (GST/receptor) fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, MO) or glutathione derivatized microtitre plates, which are then combined with the *hedgehog* polypeptide, e.g. an ³⁵S-labeled *hedgehog* polypeptide, and the test compound and incubated under conditions conducive to complex formation, e.g. at physiological conditions for salt and pH, though slightly more stringent conditions may be desired. Following incubation, the beads are washed to remove any unbound *hedgehog* polypeptide, and the matrix bead-bound radiolabel determined directly (e.g. beads placed in scintillant), or in the supernatant after the receptor/*hedgehog* complexes are dissociated. Alternatively, the complexes can be dissociated from the bead, separated by SDS-PAGE gel, and the level of *hedgehog* polypeptide found in the bead fraction quantitated from the gel using standard electrophoretic techniques.

Other techniques for immobilizing proteins on matrices are also available for use in the subject assay. For instance, soluble portions of the *hedgehog* receptor protein can be immobilized utilizing conjugation of biotin and streptavidin. For instance, biotinylated receptor molecules can be prepared from biotin-NHS (N-hydroxy-succinimide) using techniques well known in the art (e.g., biotinylation kit, Pierce Chemicals, Rockford, IL), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). Alternatively, antibodies reactive with the *hedgehog* receptor but which do not interfere with *hedgehog* binding can be derivatized to the wells of the plate, and the receptor trapped in the wells by antibody conjugation. As

above, preparations of a *hedgehog* polypeptide and a test compound are incubated in the receptor-presenting wells of the plate, and the amount of receptor/*hedgehog* complex trapped in the well can be quantitated. Exemplary methods for detecting such complexes, in addition to those described above for the GST-immobilized complexes, include immunodetection of complexes using antibodies reactive with the *hedgehog* polypeptide, or which are reactive with the receptor protein and compete for binding with the *hedgehog* polypeptide; as well as enzyme-linked assays which rely on detecting an enzymatic activity associated with the *hedgehog* polypeptide. In the instance of the latter, the enzyme can be chemically conjugated or provided as a fusion protein with the *hedgehog* polypeptide. To illustrate, the *hedgehog* polypeptide can be chemically cross-linked or genetically fused with alkaline phosphatase, and the amount of *hedgehog* polypeptide trapped in the complex can be assessed with a chromogenic substrate of the enzyme, e.g. paranitrophenylphosphate. Likewise, a fusion protein comprising the *hedgehog* polypeptide and glutathione-S-transferase can be provided, and complex formation quantitated by detecting the GST activity using 1-chloro-2,4-dinitrobenzene (Habig et al (1974) *J Biol Chem* 249:7130).

For processes which rely on immunodetection for quantitating one of the proteins trapped in the complex, antibodies against the protein, such as the anti-*hedgehog* antibodies described herein, can be used. Alternatively, the protein to be detected in the complex can be "epitope tagged" in the form of a fusion protein which includes, in addition to the *hedgehog* polypeptide or *hedgehog* receptor sequence, a second polypeptide for which antibodies are readily available (e.g. from commercial sources). For instance, the GST fusion proteins described above can also be used for quantification of binding using antibodies against the GST moiety. Other useful epitope tags include myc-epitopes (e.g., see Ellison et al. (1991) *J Biol Chem* 266:21150-21157) which includes a 10-residue sequence from c-myc, as well as the pFLAG system (International Biotechnologies, Inc.) or the pEZZ-protein A system (Pharmacia, NJ).

Where the desired portion of the *hedgehog* receptor (or other *hedgehog* binding molecule) cannot be provided in soluble form, liposomal vesicles can be used to provide manipulatable and isolatable sources of the receptor. For example, both authentic and recombinant forms of the *patched* protein can be reconstituted in artificial lipid vesicles (e.g. phosphatidylcholine liposomes) or in cell membrane-derived vesicles (see, for example, Bear et al. (1992) *Cell* 68:809-818; Newton et al. (1983) *Biochemistry* 22:6110-6117; and Reber et al. (1987) *J Biol Chem* 262:11369-11374).

In addition to cell-free assays, such as described above, the readily available source of *hedgehog* proteins provided by the art also facilitates the generation of cell-based assays for

identifying small molecule agonists/antagonists and the like. Analogous to the cell-based assays described above for screening combinatorial libraries, cells which are sensitive to *hedgehog* induction, e.g. *patched*-expressing cells or other myoblast-derived cells sensitive to *hedgehog* induction, can be contacted with a *hedgehog* protein and a test agent of interest, with the assay scoring for anything from simple binding to the cell to modulation in *hedgehog* inductive responses by the target cell in the presence and absence of the test agent. As with the cell-free assays, agents which produce a statistically significant change in *hedgehog* activities (either inhibition or potentiation) can be identified.

In other emdodiments, the cell-based assay scores for agents which disrupt association of *patched* and *smoothened* proteins, e.g., in the cell surface membrane or liposomal preparation.

In addition to characterizing cells that naturally express the *patched* protein, cells which have been genetically engineered to ectopically express *patched* can be utilized for drug screening assays. As an example, cells which either express low levels or lack expression of the *patched* protein, e.g. *Xenopus laevis* oocytes, COS cells or yeast cells, can be genetically modified using standard techniques to ectopically express the *patched* protein. (see Marigo et al., *supra*).

The resulting recombinant cells, e.g., which express a functional *patched* receptor, can be utilized in receptor binding assays to identify agonist or anatagonsts of *hedgehog* binding. Binding assays can be performed using whole cells. Furthermore, the recombinant cells of the present invention can be engineered to include other heterolgous genes encoding proteins involved in *hedgehog*-dependent siganl pathways. For example, the gene products of one or more of *smoothened*, *costal-2* and/or *fused* can be co-expressed with *patched* in the reagent cell, with assays being sensitive to the functional reconstituion of the *hedgehog* signal transduction cascade.

Alternatively, liposomal preparations using reconstituted *patched* protein can be utilized. *Patched* protein purified from detergent extracts from both authentic and recombinant origins can be reconstituted in in artificial lipid vesicles (e.g. phosphatidylcholine liposomes) or in cell membrane-derived vesicles (see, for example, Bear et al. (1992) *Cell* 68:809-818; Newton et al. (1983) *Biochemistry* 22:6110-6117; and Reber et al. (1987) *J Biol Chem* 262:11369-11374). The lamellar structure and size of the resulting liposomes can be characterized using electron microscopy. External orientation of the *patched* protein in the reconstituted membranes can be demonstrated, for example, by immunoelectron microscopy. The *hedgehog* protein binding activity of liposomes containing *patched* and liposomes without the protein in the presence of

candidate agents can be compared in order to identify potential modulators of the *hedgehog-patched* interaction.

The *hedgehog* protein used in these cell-based assays can be provided as a purified source (natural or recombinant in origin), or in the form of cells/tissue which express the protein and which are co-cultured with the target cells. As in the cell-free assays, where simple binding (rather than induction) is the hedgehog activity scored for in the assay, the protein can be labelled by any of the above-mentioned techniques, e.g., fluorescently, enzymatically or radioactively, or detected by immunoassay.

In addition to binding studies, functional assays can be used to identify modulators, i.e., agonists or antagonists, of *hedgehog* or *patched* activities. By detecting changes in intracellular signals, such as alterations in second messengers or gene expression, in *patched*-expressing cells contacted with a test agent, candidate agonists and antagonists to *patched* signaling can be identified.

A number of gene products have been implicated in *patched*-mediated signal transduction, including *patched*, the transcription factor *cubitus interruptus* (ci), the serine/threonine kinase *fused* (fu) and the gene products of *costal-2*, *smoothed* and *suppressor of fused*.

The interaction of a hedgehog protein with *patched* sets in motion a cascade involving the activation and inhibition of downstream effectors, the ultimate consequence of which is, in some instances, a detectable change in the transcription or translation of a gene. Potential transcriptional targets of *patched* signaling are the *patched* gene itself (Hidalgo and Ingham, 1990 *Development* 110, 291-301; Marigo et al., 1996) and the vertebrate homologs of the drosophila cubitus interruptus gene, the *GLI* genes (Hui et al. (1994) *Dev Biol* 162:402-413). *Patched* gene expression has been shown to be induced in cells of the limb bud and the neural plate that are responsive to *Shh*. (Marigo et al. (1996) *PNAS*, in press; Marigo et al. (1996) *Development* 122:1225-1233). The *GLI* genes encode putative transcription factors having zinc finger DNA binding domains (Orenic et al. (1990) *Genes & Dev* 4:1053-1067; Kinzler et al. (1990) *Mol Cell Biol* 10:634-642). Transcription of the *GLI* gene has been reported to be upregulated in response to *hedgehog* in limb buds, while transcription of the *GLI3* gene is downregulated in response to *hedgehog* induction (Marigo et al. (1996) *Development* 122:1225-1233). By selecting transcriptional regulatory sequences from such target genes, e.g. from *patched* or *GLI* genes, that are responsible for the up- or down regulation of these genes in response to *patched* signalling, and operatively linking such promoters to a reporter gene, one can derive a transcription based assay which is sensitive to the ability of a specific test compound

to modify *patched* signalling pathways. Expression of the reporter gene, thus, provides a valuable screening tool for the development of compounds that act as agonists or antagonists of *ptc* induction of differentiation/quiescence.

Reporter gene based assays of this invention measure the end stage of the above described cascade of events, e.g., transcriptional modulation. Accordingly, in practicing one embodiment of the assay, a reporter gene construct is inserted into the reagent cell in order to generate a detection signal dependent on *ptc* signaling. To identify potential regulatory elements responsive to *ptc* signaling present in the transcriptional regulatory sequence of a target gene, nested deletions of genomic clones of the target gene can be constructed using standard techniques. See, for example, Current Protocols in Molecular Biology, Ausubel, F.M. et al. (eds.) Greene Publishing Associates, (1989); U.S. Patent 5,266,488; Sato et al. (1995) *J Biol Chem* 270:10314-10322; and Kube et al. (1995) *Cytokine* 7:1-7. A nested set of DNA fragments from the gene's 5'-flanking region are placed upstream of a reporter gene, such as the luciferase gene, and assayed for their ability to direct reporter gene expression in *patched* expressing cells. Host cells transiently transfected with reporter gene constructs can be scored for the induction of expression of the reporter gene in the presence and absence of *hedgehog* to determine regulatory sequences which are responsive to *patched*-dependent signalling.

In practicing one embodiment of the assay, a reporter gene construct is inserted into the reagent cell in order to generate a detection signal dependent on second messengers generated by induction with *hedgehog* protein. Typically, the reporter gene construct will include a reporter gene in operative linkage with one or more transcriptional regulatory elements responsive to the *hedgehog* activity, with the level of expression of the reporter gene providing the *hedgehog*-dependent detection signal. The amount of transcription from the reporter gene may be measured using any method known to those of skill in the art to be suitable. For example, mRNA expression from the reporter gene may be detected using RNase protection or RNA-based PCR, or the protein product of the reporter gene may be identified by a characteristic stain or an intrinsic activity. The amount of expression from the reporter gene is then compared to the amount of expression in either the same cell in the absence of the test compound (or *hedgehog*) or it may be compared with the amount of transcription in a substantially identical cell that lacks the target receptor protein. Any statistically or otherwise significant difference in the amount of transcription indicates that the test compound has in some manner altered the signal transduction of the *patched* protein, e.g., the test compound is a potential *ptc* therapeutic.

As described in further detail below, in preferred embodiments the gene product of the reporter is detected by an intrinsic activity associated with that product. For instance, the reporter gene may encode a gene product that, by enzymatic activity, gives rise to a detection

signal based on color, fluorescence, or luminescence. In other preferred embodiments, the reporter or marker gene provides a selective growth advantage, e.g., the reporter gene may enhance cell viability, relieve a cell nutritional requirement, and/or provide resistance to a drug.

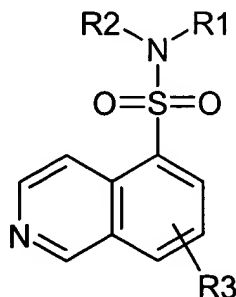
Preferred reporter genes are those that are readily detectable. The reporter gene may also be included in the construct in the form of a fusion gene with a gene that includes desired transcriptional regulatory sequences or exhibits other desirable properties. Examples of reporter genes include, but are not limited to CAT (chloramphenicol acetyl transferase) (Alton and Vapnek (1979), Nature 282: 864-869) luciferase, and other enzyme detection systems, such as beta-galactosidase; firefly luciferase (deWet et al. (1987), Mol. Cell. Biol. 7:725-737); bacterial luciferase (Engebrecht and Silverman (1984), PNAS 1: 4154-4158; Baldwin et al. (1984), Biochemistry 23: 3663-3667); alkaline phosphatase (Toh et al. (1989) Eur. J. Biochem. 182: 231-238, Hall et al. (1983) J. Mol. Appl. Gen. 2: 101), human placental secreted alkaline phosphatase (Cullen and Malim (1992) Methods in Enzymol. 216:362-368).

Transcriptional control elements which may be included in a reporter gene construct include, but are not limited to, promoters, enhancers, and repressor and activator binding sites. Suitable transcriptional regulatory elements may be derived from the transcriptional regulatory regions of genes whose expression is induced after modulation of a *patched* signal transduction pathway. The characteristics of preferred genes from which the transcriptional control elements are derived include, but are not limited to, low or undetectable expression in quiescent cells, rapid induction at the transcriptional level within minutes of extracellular stimulation, induction that is transient and independent of new protein synthesis, subsequent shut-off of transcription requires new protein synthesis, and mRNAs transcribed from these genes have a short half-life. It is not necessary for all of these properties to be present.

In yet other embodiments, second messenger generation can be measured directly in the detection step, such as mobilization of intracellular calcium, phospholipid metabolism or adenylate cyclase activity are quantitated, for instance, the products of phospholipid hydrolysis IP₃, DAG or cAMP could be measured. For example, recent studies have implicated protein kinase A (PKA) as a possible component of *hedgehog/patched* signaling (Hammerschmidt et al. (1996) *Genes & Dev* 10:647). High PKA activity has been shown to antagonize *hedgehog* signaling in these systems. Although it is unclear whether PKA acts directly downstream or in parallel with *hedgehog* signaling, it is possible that *hedgehog* signalling occurs via inhibition of PKA activity. Thus, detection of PKA activity provides a potential readout for the instant assays.

In a preferred embodiment, the *ptc* therapeutic is a PKA inhibitor. A variety of PKA inhibitors are known in the art, including both peptidyl and organic compounds. For instance,

the *ptc* therapeutic can be a 5-isoquinolinesulfonamide, such as represented in the general formula:



wherein,

R₁ and R₂ each can independently represent hydrogen, and as valence and stability permit a lower alkyl, a lower alkenyl, a lower alkynyl, a carbonyl (such as a carboxyl, an ester, a formate, or a ketone), a thiocarbonyl (such as a thioester, a thioacetate, or a thioformate), an amino, an acylamino, an amido, a cyano, a nitro, an azido, a sulfate, a sulfonate, a sulfonamido, -(CH₂)_m-R₈, -(CH₂)_m-OH, -(CH₂)_m-O-lower alkyl, -(CH₂)_m-O-lower alkenyl, -(CH₂)_n-O-(CH₂)_m-R₈, -(CH₂)_m-SH, -(CH₂)_m-S-lower alkyl, -(CH₂)_m-S-lower alkenyl, -(CH₂)_n-S-(CH₂)_m-R₈, or

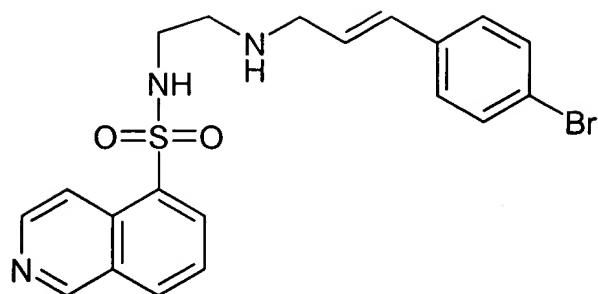
R₁ and R₂ taken together with N form a heterocycle (substituted or unsubstituted);

R₃ is absent or represents one or more substitutions to the isoquinoline ring such as a lower alkyl, a lower alkenyl, a lower alkynyl, a carbonyl (such as a carboxyl, an ester, a formate, or a ketone), a thiocarbonyl (such as a thioester, a thioacetate, or a thioformate), an amino, an acylamino, an amido, a cyano, a nitro, an azido, a sulfate, a sulfonate, a sulfonamido, -(CH₂)_m-R₈, -(CH₂)_m-OH, -(CH₂)_m-O-lower alkyl, -(CH₂)_m-O-lower alkenyl, -(CH₂)_n-O-(CH₂)_m-R₈, -(CH₂)_m-SH, -(CH₂)_m-S-lower alkyl, -(CH₂)_m-S-lower alkenyl, -(CH₂)_n-S-(CH₂)_m-R₈;

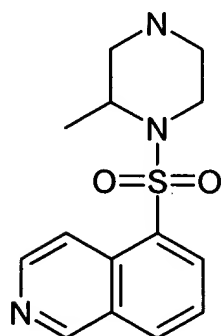
R₈ represents a substituted or unsubstituted aryl, aralkyl, cycloalkyl, cycloalkenyl, or heterocycle; and

n and m are independently for each occurrence zero or an integer in the range of 1 to 6.

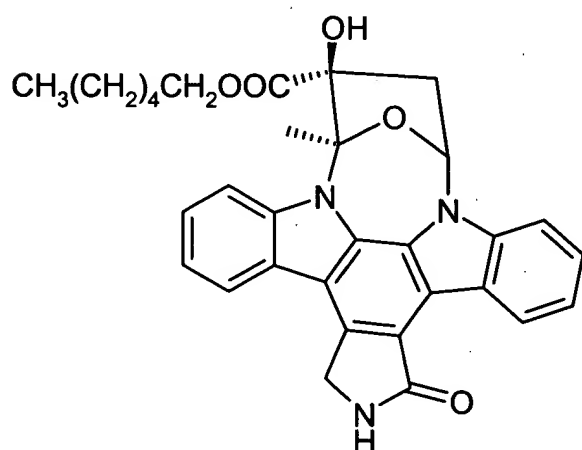
In a preferred embodiment, the PKA inhibitor is N-[2-((p-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide (H-89; Calbiochem Cat. No. 371963), e.g., having the formula:



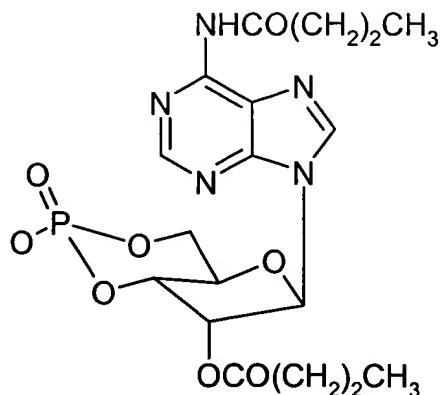
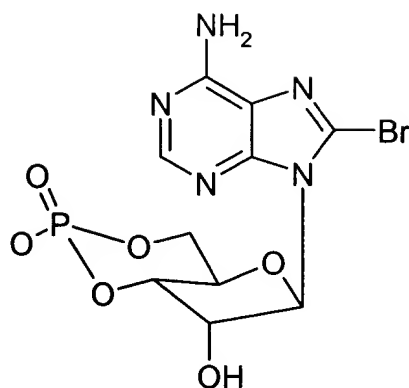
In another embodiment, the PKA inhibitor is 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7; Calbiochem Cat. No. 371955), e.g., having the formula:



- 5 In still other embodiments, the PKA inhibitor is KT5720 (Calbiochem Cat. No. 420315), having the structure



A variety of nucleoside analogs are also useful as PKA inhibitors. For example, the subject method can be carried out cyclic AMP analogs which inhibit the kinase activity of PKA, as for example, 8-bromo-cAMP or dibutyryl-cAMP



Exemplary peptidyl inhibitors of PKA activity include the PKA Heat Stable Inhibitor (isoform α ; see, for example, Calbiochem Cat. No. 539488, and Wen et al. (1995) *J Biol Chem* 270:2041).

Certain *hedgehog* receptors may stimulate the activity of phospholipases. Inositol lipids can be extracted and analyzed using standard lipid extraction techniques. Water soluble derivatives of all three inositol lipids (IP₁, IP₂, IP₃) can also be quantitated using radiolabelling techniques or HPLC.

The mobilization of intracellular calcium or the influx of calcium from outside the cell may be a response to *hedgehog* stimulation or lack thereof. Calcium flux in the reagent cell can be measured using standard techniques. The choice of the appropriate calcium indicator, fluorescent, bioluminescent, metallochromic, or Ca⁺⁺-sensitive microelectrodes depends on the cell type and the magnitude and time constant of the event under study (Borle (1990) *Environ. Health Perspect* 84:45-56). As an exemplary method of Ca⁺⁺ detection, cells could be loaded with the Ca⁺⁺ sensitive fluorescent dye fura-2 or indo-1, using standard methods, and any change in Ca⁺⁺ measured using a fluorometer.

In certain embodiments of the assay, it may be desirable to screen for changes in cellular phosphorylation. As an example, the drosophila gene *fused* (fu) which encodes a serine/threonine kinase has been identified as a potential downstream target in *hedgehog* signaling. (Preat et al., 1990 *Nature* 347, 87-89; Therond et al. 1993, *Mech. Dev.* 44. 65-80). The ability of compounds to modulate serine/threonine kinase activation could be screened using colony immunoblotting (Lyons and Nelson (1984) *Proc. Natl. Acad. Sci. USA* 81:7426-7430) using antibodies against phosphorylated serine or threonine residues. Reagents for performing such assays are commercially available, for example, phosphoserine and phosphothreonine specific antibodies which measure increases in phosphorylation of those residues can be purchased from commercial sources.

In yet another embodiment, the *ptc* therapeutic is an antisense molecule which inhibits expression of a protein involved in a *patched*-mediated signal transduction pathway. To illustrate, by inhibiting the expression of a protein which are involved in *patched* signals, such as fused, costal-2, smoothened and/or Gli genes, the ability of the patched signal pathway(s) to inhibit proliferation of a cell can be altered, e.g., potentiated or repressed.

As used herein, "antisense" therapy refers to administration or *in situ* generation of oligonucleotide probes or their derivatives which specifically hybridize (e.g. bind) under cellular conditions with cellular mRNA and/or genomic DNA encoding a *hedgehog* protein, patched, or a protein involved in patched-mediated signal transduction. The hybridization should inhibit expression of that protein, e.g. by inhibiting transcription and/or translation. The binding may be by conventional base pair complementarity, or, for example, in the case of binding to DNA duplexes, through specific interactions in the major groove of the double helix. In general, "antisense" therapy refers to the range of techniques generally employed in the art, and includes any therapy which relies on specific binding to oligonucleotide sequences.

An antisense construct of the present invention can be delivered, for example, as an expression plasmid which, when transcribed in the cell, produces RNA which is complementary to at least a unique portion of the target cellular mRNA. Alternatively, the antisense construct is an oligonucleotide probe which is generated *ex vivo* and which, when introduced into the cell causes inhibition of expression by hybridizing with the mRNA and/or genomic sequences of a target gene. Such oligonucleotide probes are preferably modified oligonucleotide which are resistant to endogenous nucleases, e.g. exonucleases and/or endonucleases, and is therefore stable *in vivo*. Exemplary nucleic acid molecules for use as antisense oligonucleotides are phosphoramidate, phosphothioate and methylphosphonate analogs of DNA (see also U.S. Patents 5,176,996; 5,264,564; and 5,256,775). Additionally, general approaches to constructing oligomers useful in antisense therapy have been reviewed, for example, by Van der Krol et al. (1988) *Biotechniques* 6:958-976; and Stein et al. (1988) *Cancer Res* 48:2659-2668.

Several considerations should be taken into account when constructing antisense oligonucleotides for the use in the methods of the invention: (1) oligos should have a GC content of 50% or more; (2) avoid sequences with stretches of 3 or more G's; and (3) oligonucleotides should not be longer than 25-26 mers. When testing an antisense oligonucleotide, a mismatched control can be constructed. The controls can be generated by reversing the sequence order of the corresponding antisense oligonucleotide in order to conserve the same ratio of bases.

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In an illustrative embodiment, the *ptc* therapeutic can be an antisense construct for inhibiting the expression of *patched*, e.g., to mimic the inhibition of *patched* by *hedgehog*. Exemplary antisense constructs include:

5'-GTCCTGGCGCCGCCGCCGCCGTCGCC

5 5'-TTCCGATGACCGGCCTTTCGCGGTGA

5'-GTGCACGGAAAGGTGCAGGCCACACT

VI. Exemplary pharmaceutical preparations of *hedgehog* and *ptc* therapeutics

The source of the *hedgehog* and *ptc* therapeutics to be formulated will depend on the particular form of the agent. Small organic molecules and peptidyl fragments can be chemically synthesized and provided in a pure form suitable for pharmaceutical/cosmetic usage. Products of natural extracts can be purified according to techniques known in the art. For example, the Cox et al. U.S. Patent 5,286,654 describes a method for purifying naturally occurring forms of a secreted protein and can be adapted for purification of *hedgehog* polypeptides. Recombinant sources of *hedgehog* polypeptides are also available. For example, the gene encoding *hedgehog* polypeptides, are known, *inter alia*, from PCT publications WO 95/18856 and WO 96/17924.

Those of skill in treating peripheral neuropathies can determine the effective amount of an *hedgehog* or *ptc* therapeutic to be formulated in a pharmaceutical or cosmetic preparation.

The *hedgehog* or *ptc* therapeutic formulations used in the method of the invention are most preferably applied in the form of appropriate compositions. As appropriate compositions there may be cited all compositions usually employed for systemically or topically administering drugs. The pharmaceutically acceptable carrier should be substantially inert, so as not to act with the active component. Suitable inert carriers include water, alcohol polyethylene glycol, mineral oil or petroleum gel, propylene glycol and the like.

To prepare the pharmaceutical compositions of this invention, an effective amount of the particular *hedgehog* or *ptc* therapeutic as the active ingredient is combined in intimate admixture with a pharmaceutically acceptable carrier, which carrier may take a wide variety of forms depending on the form of preparation desired for administration. These pharmaceutical compositions are desirable in unitary dosage form suitable, particularly, for administration orally, rectally, percutaneously, or by parenteral injection. For example, in preparing the compositions in oral dosage form, any of the usual pharmaceutical media may be employed such as, for example, water, glycols, oils, alcohols and the like in the case of oral liquid preparations such as

suspensions, syrups, elixirs and solutions; or solid carriers such as starches, sugars, kaolin, lubricants, binders, disintegrating agents and the like in the case of powders, pills, capsules, and tablets. Because of their ease in administration, tablets and capsules represents the most advantageous oral dosage unit form, in which case solid pharmaceutical carriers are obviously employed. For parenteral compositions, the carrier will usually comprise sterile water, at least in large part, though other ingredients, for example, to aid solubility, may be included. Injectable solutions, for example, may be prepared in which the carrier comprises saline solution, glucose solution or a mixture of saline and glucose solution. Injectable suspensions may also be prepared in which case appropriate liquid carriers, suspending agents and the like may be employed. Also included are solid form preparations which are intended to be converted, shortly before use, to liquid form preparations. In the compositions suitable for percutaneous administration, the carrier optionally comprises a penetration enhancing agent and/or a suitable wetting agent, optionally combined with suitable additives of any nature in minor proportions, which additives do not introduce a significant deleterious effect on the skin.

In addition to the direct topical application of the preparations they can be topically administered by other methods, for example, encapsulated in a temperature and/or pressure sensitive matrix or in film or solid carrier which is soluble in body fluids and the like for subsequent release, preferably sustained-release of the active component.

As appropriate compositions for topical application there may be cited all compositions usually employed for topically administering therapeutics, e.g., creams, gellies, dressings, shampoos, tinctures, pastes, ointments, salves, powders, liquid or semiliquid formulation and the like. Application of said compositions may be by aerosol e.g. with a propellant such as nitrogen carbon dioxide, a freon, or without a propellant such as a pump spray, drops, lotions, or a semisolid such as a thickened composition which can be applied by a swab. In particular compositions, semisolid compositions such as salves, creams, pastes, gellies, ointments and the like will conveniently be used.

It is especially advantageous to formulate the subject compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used in the specification and claims herein refers to physically discrete units suitable as unitary dosages, each unit containing a predetermined quantity of active ingredient calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. Examples of such dosage unit forms are tablets (including scored or coated tablets), capsules, pills, powders packets, wafers, injectable solutions or suspensions, teaspoonfuls, tablespoonfuls and the like, and segregated multiples thereof.

The pharmaceutical preparations of the present invention can be used, as stated above, for the many applications which can be considered cosmetic uses. Cosmetic compositions known in the art, preferably hypoallergic and pH controlled are especially preferred, and include toilet waters, packs, lotions, skin milks or milky lotions. The preparations contain, besides the hedgehog or ptc therapeutic, components usually employed in such preparations. Examples of such components are oils, fats, waxes, surfactants, humectants, thickening agents, antioxidants, viscosity stabilizers, chelating agents, buffers, preservatives, perfumes, dyestuffs, lower alkanols, and the like. If desired, further ingredients may be incorporated in the compositions, e.g. antiinflammatory agents, antibacterials, antifungals, disinfectants, vitamins, sunscreens, antibiotics, or other anti-acne agents.

Examples of oils comprise fats and oils such as olive oil and hydrogenated oils; waxes such as beeswax and lanolin; hydrocarbons such as liquid paraffin, ceresin, and squalane; fatty acids such as stearic acid and oleic acid; alcohols such as cetyl alcohol, stearyl alcohol, lanolin alcohol, and hexadecanol; and esters such as isopropyl myristate, isopropyl palmitate and butyl stearate. As examples of surfactants there may be cited anionic surfactants such as sodium stearate, sodium cetylsulfate, polyoxyethylene laurylether phosphate, sodium N-acyl glutamate; cationic surfactants such as stearyldimethylbenzylammonium chloride and stearyltrimethylammonium chloride; ampholytic surfactants such as alkylaminoethylglycine hydrochloride solutions and lecithin; and nonionic surfactants such as glycerin monostearate, sorbitan monostearate, sucrose fatty acid esters, propylene glycol monostearate, polyoxyethylene oleylether, polyethylene glycol monostearate, polyoxyethylene sorbitan monopalmitate, polyoxyethylene coconut fatty acid monoethanolamide, polyoxypropylene glycol (e.g. the materials sold under the trademark "Pluronic"), polyoxyethylene castor oil, and polyoxyethylene lanolin. Examples of humectants include glycerin, 1,3-butylene glycol, and propylene glycol; examples of lower alcohols include ethanol and isopropanol; examples of thickening agents include xanthan gum, hydroxypropyl cellulose, hydroxypropyl methyl cellulose, polyethylene glycol and sodium carboxymethyl cellulose; examples of antioxidants comprise butylated hydroxytoluene, butylated hydroxyanisole, propyl gallate, citric acid and ethoxyquin; examples of chelating agents include disodium edetate and ethanedihydroxy diphosphate; examples of buffers comprise citric acid, sodium citrate, boric acid, borax, and disodium hydrogen phosphate; and examples of preservatives are methyl parahydroxybenzoate, ethyl parahydroxybenzoate, dehydroacetic acid, salicylic acid and benzoic acid.

For preparing ointments, creams, toilet waters, skin milks, and the like, typically from 0.01 to 10% in particular from 0.1 to 5% and more in particular from 0.2 to 2.5% of the active ingredient, e.g., of the hedgehog or ptc therapeutic, will be incorporated in the compositions. In

ointments or creams, the carrier for example consists of 1 to 20%, in particular 5 to 15% of a humectant, 0.1 to 10% in particular from 0.5 to 5% of a thickener and water; or said carrier may consist of 70 to 99%, in particular 20 to 95% of a surfactant, and 0 to 20%, in particular 2.5 to 15% of a fat; or 80 to 99.9% in particular 90 to 99% of a thickener; or 5 to 15% of a surfactant, 2-15% of a humectant, 0 to 80% of an oil, very small ($< 2\%$) amounts of preservative, coloring agent and/or perfume, and water. In a toilet water, the carrier for example consists of 2 to 10% of a lower alcohol, 0.1 to 10% or in particular 0.5 to 1% of a surfactant, 1 to 20%, in particular 3 to 7% of a humectant, 0 to 5% of a buffer, water and small amounts ($< 2\%$) of preservative, dyestuff and/or perfume. In a skin milk, the carrier typically consists of 10-50% of oil, 1 to 10% of surfactant, 50-80% of water and 0 to 3% of preservative and/or perfume. In the aforementioned preparations, all % symbols refer to weight by weight percentage.

Particular compositions for use in the method of the present invention are those wherein the hedgehog or ptc therapeutic is formulated in liposome-containing compositions. Liposomes are artificial vesicles formed by amphiphatic molecules such as polar lipids, for example, phosphatidyl cholines, ethanolamines and serines, sphingomyelins, cardiolipins, plasmalogens, phosphatidic acids and cerebiosides. Liposomes are formed when suitable amphiphathic molecules are allowed to swell in water or aqueous solutions to form liquid crystals usually of multilayer structure comprised of many bilayers separated from each other by aqueous material (also referred to as coarse liposomes). Another type of liposome known to be consisting of a single bilayer encapsulating aqueous material is referred to as a unilamellar vesicle. If water-soluble materials are included in the aqueous phase during the swelling of the lipids they become entrapped in the aqueous layer between the lipid bilayers.

Water-soluble active ingredients such as, for example, various salt forms of a hedgehog polypeptide, are encapsulated in the aqueous spaces between the molecular layers. The lipid soluble active ingredient of hedgehog or ptc therapeutic, such as an organic mimetic, is predominantly incorporated into the lipid layers, although polar head groups may protude from the layer into the aqueous space. The encapsulation of these compounds can be achieved by a number of methods. The method most commonly used involves casting a thin film of phospholipid onto the walls of a flask by evaporation from an organic solvent. When this film is dispersed in a suitable aqueous medium, multilamellar liposomes are formed. Upon suitable sonication, the coarse liposomes form smaller similarly closed vesicles.

Water-soluble active ingredients are usually incorporated by dispersing the cast film with an aqueous solution of the compound. The unencapsulated compound is then removed by centrifugation, chromatography, dialysis or other art-known suitable procedures. The lipid-

soluble active ingredient is usually incorporated by dissolving it in the organic solvent with the phospholipid prior to casting the film. If the solubility of the material in the lipid phase is not exceeded or the amount present is not in excess of that which can be bound to the lipid, liposomes prepared by the above method usually contain most of the material bound in the lipid bilayers; separation of the liposomes from unencapsulated material is not required.

A particularly convenient method for preparing liposome formulated forms of hedgehog and ptc therapeutics is the method described in EP-A-253,619, incorporated herein by reference. In this method, single bilayered liposomes containing encapsulated active ingredients are prepared by dissolving the lipid component in an organic medium, injecting the organic solution of the lipid component under pressure into an aqueous component while simultaneously mixing the organic and aqueous components with a high speed homogenizer or mixing means, whereupon the liposomes are formed spontaneously.

The single bilayered liposomes containing the encapsulated hedgehog or ptc therapeutic can be employed directly or they can be employed in a suitable pharmaceutically acceptable carrier for topical administration. The viscosity of the liposomes can be increased by the addition of one or more suitable thickening agents such as, for example xanthan gum, hydroxypropyl cellulose, hydroxypropyl methylcellulose and mixtures thereof. The aqueous component may consist of water alone or it may contain electrolytes, buffered systems and other ingredients, such as, for example, preservatives. Suitable electrolytes which can be employed include metal salts such as alkali metal and alkaline earth metal salts. The preferred metal salts are calcium chloride, sodium chloride and potassium chloride. The concentration of the electrolyte may vary from zero to 260 mM, preferably from 5 mM to 160 mM. The aqueous component is placed in a suitable vessel which can be adapted to effect homogenization by effecting great turbulence during the injection of the organic component. Homogenization of the two components can be accomplished within the vessel, or, alternatively, the aqueous and organic components may be injected separately into a mixing means which is located outside the vessel. In the latter case, the liposomes are formed in the mixing means and then transferred to another vessel for collection purpose.

The organic component consists of a suitable non-toxic, pharmaceutically acceptable solvent such as, for example ethanol, glycerol, propylene glycol and polyethylene glycol, and a suitable phospholipid which is soluble in the solvent. Suitable phospholipids which can be employed include lecithin, phosphatidylcholine, phosphatidylserine, phosphatidylethanol-amine, phosphatidylinositol, lysophosphatidylcholine and phosphatidyl glycerol, for example. Other lipophilic additives may be employed in order to selectively modify the characteristics of the

liposomes. Examples of such other additives include stearylamine, phosphatidic acid, tocopherol, cholesterol and lanolin extracts.

In addition, other ingredients which can prevent oxidation of the phospholipids may be added to the organic component. Examples of such other ingredients include tocopherol, butylated hydroxyanisole, butylated hydroxytoluene, ascorbyl palmitate and ascorbyl oleate. Preservatives such as benzoic acid, methyl paraben and propyl paraben may also be added.

Apart from the above-described compositions, use may be made of covers, e.g. plasters, bandages, dressings, gauze pads and the like, containing an appropriate amount of a hedgehog or ptc therapeutic. In some cases use may be made of plasters, bandages, dressings, gauze pads and the like which have been impregnated with a topical formulation containing the therapeutic formulation.

VII. Exemplary Hedgehog Antagonists

Exemplary *hedgehog* antagonists of the invention include cyclopamine, tomatidine, jervine, AY9944, triparanol, forskolin, cAMP, dibutyryl cAMP (and other hydrophobically modified cAMP variants) and functionally effective derivatives thereof. Additional *hedgehog* antagonists, functionally effective derivatives, compositions and methods for making such compounds are described in detail in the following U.S. Patent applications: Baxter et al., 09/663,835; Beachy et al. entitled "Inhibitors of hedgehog signaling pathways, compositions and uses related thereto" filed October 10, 2000; Baxter et al. "Mediators of hedgehog signaling pathway, compositions and uses related thereto" filed October 13, 2000; Philip Beachy, "Regulators of the hedgehog pathway, compositions and uses related thereto" filed October 13, 2000.

Evaluation of the effects of hedgehog on T-cell development

The invention now being generally described, it will be more readily understood by reference to the following examples which are included merely for purposes of illustration of certain aspects and embodiments of the present invention, and are not intended to limit the invention.

Utilizing a fetal thymic organ culture, the effects of *Shh* on T-cell maturation was determined. Briefly, immature thymocytes (CD4-/CD8- [DN]) were isolated from E14 thymus.

The cells were culture for about 7 days, and the level of mature thymocytes, e.g., CD4+/CD8+ [DP], was assessed. In certain cultures, octyl-modified Shh was added to the culture; in other cultures, the anti-hedgehog 5E1 antibody was added. As illustrated in Figures 1-4, the addition of Shh to the culture resulted in an increase in the percentage of cells after 7 days with markers indicative of immature thymocytes. Conversely, addition of the 5E1 antibody resulted in an increase in the percentage of mature T lymphocytes after 7 days.

All of the above-cited references and publications are hereby incorporated by reference.

10 **Equivalents**

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific polypeptides, nucleic acids, methods, assays and reagents described herein. Such equivalents are considered to be within the scope of this invention.

SEQUENCE LISTING

- 5 (2) INFORMATION FOR SEQ ID NO:1:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1277 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: both
 10 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

- 15 (ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 1..1275

- 20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

25	ATG GTC GAA ATG CTG CTG TTG ACA AGA ATT CTC TTG GTG GGC TTC ATC	48
	Met Val Glu Met Leu Leu Leu Thr Arg Ile Leu Leu Val Gly Phe Ile	
	1 5 10 15	
30	TGC GCT CTT TTA GTC TCC TCT GGG CTG ACT TGT GGA CCA GGC AGG GGC	96
	Cys Ala Leu Leu Val Ser Ser Gly Leu Thr Cys Gly Pro Gly Arg Gly	
	20 25 30	
35	ATT GGA AAA AGG AGG CAC CCC AAA AAG CTG ACC CCG TTA GCC TAT AAG	144
	Ile Gly Lys Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys	
	35 40 45	
40	CAG TTT ATT CCC AAT GTG GCA GAG AAG ACC CTA GGG GCC AGT GGA AGA	192
	Gln Phe Ile Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Arg	
	50 55 60	
45	TAT GAA GGG AAG ATC ACA AGA AAC TCC GAG AGA TTT AAA GAA CTA ACC	240
	Tyr Glu Gly Lys Ile Thr Arg Asn Ser Glu Arg Phe Lys Glu Leu Thr	
	65 70 75 80	
50	CCA AAT TAC AAC CCT GAC ATT ATT TTT AAG GAT GAA GAG AAC ACG GGA	288
	Pro Asn Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Gly	
	85 90 95	
55	GCT GAC AGA CTG ATG ACT CAG CGC TGC AAG GAC AAG CTG AAT GCC CTG	336
	Ala Asp Arg Leu Met Thr Gln Arg Cys Lys Asp Lys Leu Asn Ala Leu	
	100 105 110	
60	GCG ATC TCG GTG ATG AAC CAG TGG CCC GGG GTG AAG CTG CGG GTG ACC	384
	Ala Ile Ser Val Met Asn Gln Trp Pro Gly Val Lys Leu Arg Val Thr	
	115 120 125	
65	GAG GGC TGG GAC GAG GAT GGC CAT CAC TCC GAG GAA TCG CTG CAC TAC	432
	Glu Gly Trp Asp Glu Asp Gly His His Ser Glu Glu Ser Leu His Tyr	
	130 135 140	
70	GAG GGT CGC GCC GTG GAC ATC ACC ACG TCG GAT CGG GAC CGC AGC AAG	480
	Glu Gly Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg Ser Lys	
	145 150 155 160	

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CTC	CTC	TAC	CGC	ATC	GGC	AGC	TGG	GTG	CTG	GAT	GGT	GAC	GCG	CTG	CAT	1248
Leu	Leu	Tyr	Arg	Ile	Gly	Ser	Trp	Val	Leu	Asp	Gly	Asp	Ala	Leu	His	
				405					410					415		

5	CCG	CTG	GGC	ATG	GTG	GCA	CCG	GCC	AGC	TG	1277
	Pro	Leu	Gly	Met	Val	Ala	Pro	Ala	Ser		
				420					425		

10 (2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1190 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: both
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 1..1191

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

30	ATG	GCT	CTG	CCG	GCC	AGT	CTG	TTG	CCC	CTG	TGC	TGC	TTG	GCA	CTC	TTG	48
	Met	Ala	Leu	Pro	Ala	Ser	Leu	Leu	Pro	Leu	Cys	Cys	Leu	Ala	Leu	Leu	
	1				5					10					15		
35	GCA	CTA	TCT	GCC	CAG	AGC	TGC	GGG	CCG	GGC	CGA	GGA	CCG	GTT	GGC	CGG	96
	Ala	Leu	Ser	Ala	Gln	Ser	Cys	Gly	Pro	Gly	Arg	Gly	Pro	Val	Gly	Arg	
				20					25					30			
40	CGG	CGT	TAT	GTG	CGC	AAG	CAA	CTT	GTG	CCT	CTG	CTA	TAC	AAG	CAG	TTT	144
	Arg	Arg	Tyr	Val	Arg	Lys	Gln	Leu	Val	Pro	Leu	Leu	Tyr	Lys	Gln	Phe	
			35				40						45				
45	GTG	CCC	AGT	ATG	CCC	GAG	CGG	ACC	CTG	GGC	GCG	AGT	GGG	CCA	GCG	GAG	192
	Val	Pro	Ser	Met	Pro	Glu	Arg	Thr	Leu	Gly	Ala	Ser	Gly	Pro	Ala	Glu	
		50				55					60						
50	GGG	AGG	GTA	ACA	AGG	GGG	TCG	GAG	CGC	TTC	CGG	GAC	CTC	GTA	CCC	AAC	240
	Gly	Arg	Val	Thr	Arg	Gly	Ser	Glu	Arg	Phe	Arg	Asp	Leu	Val	Pro	Asn	
	65				70					75					80		
55	TAC	AAC	CCC	GAC	ATA	ATC	TTC	AAG	GAT	GAG	GAG	AAC	AGC	GGC	GCA	GAC	288
	Tyr	Asn	Pro	Asp	Ile	Ile	Phe	Lys	Asp	Glu	Glu	Asn	Ser	Gly	Ala	Asp	
				85					90					95			
60	CGC	CTG	ATG	ACA	GAG	CGT	TGC	AAA	GAG	CGG	GTG	AAC	GCT	CTA	GCC	ATC	336
	Arg	Leu	Met	Thr	Glu	Arg	Cys	Lys	Glu	Arg	Val	Asn	Ala	Leu	Ala	Ile	
				100				105						110			
65	GCG	GTG	ATG	AAC	ATG	TGG	CCC	GGA	GTA	CGC	CTA	CGT	GTG	ACT	GAA	GGC	384
	Ala	Val	Met	Asn	Met	Trp	Pro	Gly	Val	Arg	Leu	Arg	Val	Thr	Glu	Gly	
			115					120					125				

60

CTG	CTC	CCT	GGG	GGT	GCA	GTC	CAG	CCG	ACT	GGC	ATG	CAT	TGG	TAC	TCT	1152
Leu	Leu	Pro	Gly	Gly	Ala	Val	Gln	Pro	Thr	Gly	Met	His	Trp	Tyr	Ser	
370						375					380					

5	CGC	CTC	CTT	TAC	CGC	TTG	GCC	GAG	GAG	TTA	ATG	GGC	TG	1190
	Arg	Leu	Leu	Tyr	Arg	Leu	Ala	Glu	Glu	Leu	Met	Gly		
	385					390					395			

10 (2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 1281 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..1233

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATG	TCT	CCC	GCC	TGG	CTC	CGG	CCC	CGA	CTG	CGG	TTC	TGT	CTG	TTC	CTG	48
Met	Ser	Pro	Ala	Trp	Leu	Arg	Pro	Arg	Leu	Arg	Phe	Cys	Leu	Phe	Leu	
1				5					10					15		
CTG	CTG	CTG	CTT	CTG	GTG	CCG	GCG	GCG	CGG	GGC	TGC	GGG	CCG	GGC	CGG	96
Leu	Leu	Leu	Leu	Leu	Val	Pro	Ala	Ala	Arg	Gly	Cys	Gly	Pro	Gly	Arg	
			20					25					30			
GTG	GTG	GGC	AGC	CGC	CGG	AGG	CCG	CCT	CGC	AAG	CTC	GTG	CCT	CTT	GCC	144
Val	Val	Gly	Ser	Arg	Arg	Arg	Pro	Pro	Arg	Lys	Leu	Val	Pro	Leu	Ala	
		35					40					45				
TAC	AAG	CAG	TTC	AGC	CCC	AAC	GTG	CCG	GAG	AAG	ACC	CTG	GGC	GCC	AGC	192
Tyr	Lys	Gln	Phe	Ser	Pro	Asn	Val	Pro	Glu	Lys	Thr	Leu	Gly	Ala	Ser	
	50					55					60					
GGG	CGC	TAC	GAA	GGC	AAG	ATC	GCG	CGC	AGC	TCT	GAG	CGC	TTC	AAA	GAG	240
Gly	Arg	Tyr	Glu	Gly	Lys	Ile	Ala	Arg	Ser	Ser	Glu	Arg	Phe	Lys	Glu	
65					70					75					80	
CTC	ACC	CCC	AAC	TAC	AAT	CCC	GAC	ATC	ATC	TTC	AAG	GAC	GAG	GAG	AAC	288
Leu	Thr	Pro	Asn	Tyr	Asn	Pro	Asp	Ile	Ile	Phe	Lys	Asp	Glu	Glu	Asn	
			85					90						95		
ACG	GGT	GCC	GAC	CGC	CTC	ATG	ACC	CAG	CGC	TGC	AAG	GAC	CGT	CTG	AAC	336
Thr	Gly	Ala	Asp	Arg	Leu	Met	Thr	Gln	Arg	Cys	Lys	Asp	Arg	Leu	Asn	
		100						105					110			
TCA	CTG	GCC	ATC	TCT	GTC	ATG	AAC	CAG	TGG	CCT	GGT	GTG	AAA	CTG	CGG	384
Ser	Leu	Ala	Ile	Ser	Val	Met	Asn	Gln	Trp	Pro	Gly	Val	Lys	Leu	Arg	
		115					120					125				
GTG	ACC	GAA	GGC	CGG	GAT	GAA	GAT	GGC	CAT	CAC	TCA	GAG	GAG	TCT	TTA	432

	Val	Thr	Glu	Gly	Arg	Asp	Glu	Asp	Gly	His	His	Ser	Glu	Glu	Ser	Leu	
	130						135					140					
5	CAC	TAT	GAG	GGC	CGC	GCG	GTG	GAT	ATC	ACC	ACC	TCA	GAC	CGT	GAC	CGA	480
	His	Tyr	Glu	Gly	Arg	Ala	Val	Asp	Ile	Thr	Thr	Ser	Asp	Arg	Asp	Arg	
	145					150					155					160	
10	AAT	AAG	TAT	GGA	CTG	CTG	GCG	CGC	TTA	GCA	GTG	GAG	GCC	GGC	TTC	GAC	528
	Asn	Lys	Tyr	Gly	Leu	Leu	Ala	Arg	Leu	Ala	Val	Glu	Ala	Gly	Phe	Asp	
					165					170					175		
15	TGG	GTG	TAT	TAC	GAG	TCC	AAG	GCC	CAC	GTG	CAT	TGC	TCT	GTC	AAG	TCT	576
	Trp	Val	Tyr	Tyr	Glu	Ser	Lys	Ala	His	Val	His	Cys	Ser	Val	Lys	Ser	
					180					185				190			
20	GAG	CAT	TCG	GCC	GCT	GCC	AAG	ACA	GGT	GGC	TGC	TTT	CCT	GCC	GGA	GCC	624
	Glu	His	Ser	Ala	Ala	Ala	Lys	Thr	Gly	Gly	Cys	Phe	Pro	Ala	Gly	Ala	
			195					200					205				
25	CAG	GTG	CGC	CTA	GAG	AAC	GGG	GAG	CGT	GTG	GCC	CTG	TCA	GCT	GTA	AAG	672
	Gln	Val	Arg	Leu	Glu	Asn	Gly	Glu	Arg	Val	Ala	Leu	Ser	Ala	Val	Lys	
		210					215					220					
30	CCA	GGA	GAC	CGG	GTG	CTG	GCC	ATG	GGG	GAG	GAT	GGG	ACC	CCC	ACC	TTC	720
	Pro	Gly	Asp	Arg	Val	Leu	Ala	Met	Gly	Glu	Asp	Gly	Thr	Pro	Thr	Phe	
		225				230					235					240	
35	AGT	GAT	GTG	CTT	ATT	TTC	CTG	GAC	CGC	GAG	CCA	AAC	CGG	CTG	AGA	GCT	768
	Ser	Asp	Val	Leu	Ile	Phe	Leu	Asp	Arg	Glu	Pro	Asn	Arg	Leu	Arg	Ala	
					245					250					255		
40	TTC	CAG	GTC	ATC	GAG	ACT	CAG	GAT	CCT	CCG	CGT	CGG	CTG	GCG	CTC	ACG	816
	Phe	Gln	Val	Ile	Glu	Thr	Gln	Asp	Pro	Pro	Arg	Arg	Leu	Ala	Leu	Thr	
				260					265					270			
45	CCT	GCC	CAC	CTG	CTC	TTC	ATT	GCG	GAC	AAT	CAT	ACA	GAA	CCA	GCA	GCC	864
	Pro	Ala	His	Leu	Leu	Phe	Ile	Ala	Asp	Asn	His	Thr	Glu	Pro	Ala	Ala	
			275					280					285				
50	CAC	TTC	CGG	GCC	ACA	TTT	GCC	AGC	CAT	GTG	CAA	CCA	GGC	CAA	TAT	GTG	912
	His	Phe	Arg	Ala	Thr	Phe	Ala	Ser	His	Val	Gln	Pro	Gly	Gln	Tyr	Val	
		290					295					300					
55	CTG	GTA	TCA	GGG	GTA	CCA	GGC	CTC	CAG	CCT	GCT	CGG	GTG	GCA	GCT	GTC	960
	Leu	Val	Ser	Gly	Val	Pro	Gly	Leu	Gln	Pro	Ala	Arg	Val	Ala	Ala	Val	
		305				310					315					320	
60	TCC	ACC	CAC	GTG	GCC	CTT	GGG	TCC	TAT	GCT	CCT	CTC	ACA	AGG	CAT	GGG	1008
	Ser	Thr	His	Val	Ala	Leu	Gly	Ser	Tyr	Ala	Pro	Leu	Thr	Arg	His	Gly	
					325					330					335		
65	ACA	CTT	GTG	GTG	GAG	GAT	GTG	GTG	GCC	TCC	TGC	TTT	GCA	GCT	GTG	GCT	1056
	Thr	Leu	Val	Val	Glu	Asp	Val	Val	Ala	Ser	Cys	Phe	Ala	Ala	Val	Ala	
				340					345					350			
70	GAC	CAC	CAT	CTG	GCT	CAG	TTG	GCC	TTC	TGG	CCC	CTG	CGA	CTG	TTT	CCC	1104
	Asp	His	His	Leu	Ala	Gln	Leu	Ala	Phe	Trp	Pro	Leu	Arg	Leu	Phe	Pro	
			355					360					365				
75	AGT	TTG	GCA	TGG	GGC	AGC	TGG	ACC	CCA	AGT	GAG	GGT	GTT	CAC	TCC	TAC	1152

Ser Leu Ala Trp Gly Ser Trp Thr Pro Ser Glu Gly Val His Ser Tyr
 370 375 380

5 CCT CAG ATG CTC TAC CGC CTG GGG CGT CTC TTG CTA GAA GAG AGC ACC 1200
 Pro Gln Met Leu Tyr Arg Leu Gly Arg Leu Leu Leu Glu Glu Ser Thr
 385 390 395 400

10 TTC CAT CCA CTG GGC ATG TCT GGG GCA GGA AGC TGAAGGGACT CTAACCACTG 1253
 Phe His Pro Leu Gly Met Ser Gly Ala Gly Ser
 405 410

CCCTCCTGGA ACTGCTGTGC GTGGATCC 1281

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1313 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: both
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

- (ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 1..1314

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ATG CTG CTG CTG CTG GCC AGA TGT TTT CTG GTG ATC CTT GCT TCC TCG 48
 Met Leu Leu Leu Leu Ala Arg Cys Phe Leu Val Ile Leu Ala Ser Ser
 1 5 10 15

CTG CTG GTG TGC CCC GGG CTG GCC TGT GGG CCC GGC AGG GGG TTT GGA 96
 Leu Leu Val Cys Pro Gly Leu Ala Cys Gly Pro Gly Arg Gly Phe Gly
 20 25 30

AAG AGG CGG CAC CCC AAA AAG CTG ACC CCT TTA GCC TAC AAG CAG TTT 144
 Lys Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys Gln Phe
 35 40 45

ATT CCC AAC GTA GCC GAG AAG ACC CTA GGG GCC AGC GGC AGA TAT GAA 192
 Ile Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Arg Tyr Glu
 50 55 60

GGG AAG ATC ACA AGA AAC TCC GAA CGA TTT AAG GAA CTC ACC CCC AAT 240
 Gly Lys Ile Thr Arg Asn Ser Glu Arg Phe Lys Glu Leu Thr Pro Asn
 65 70 75 80

TAC AAC CCC GAC ATC ATA TTT AAG GAT GAG GAA AAC ACG GGA GCA GAC 288
 Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Gly Ala Asp
 85 90 95

CGG CTG ATG ACT CAG AGG TGC AAA GAC AAG TTA AAT GCC TTG GCC ATC 336
 Arg Leu Met Thr Gln Arg Cys Lys Asp Lys Leu Asn Ala Leu Ala Ile
 100 105 110

60

ATC GAG GAG CAC AGC TGG GCA CAC CGG GCC TTC GCG CCT TTC CGC CTG 1104
Ile Glu Glu His Ser Trp Ala His Arg Ala Phe Ala Pro Phe Arg Leu
355 360 365

5 GCG CAC GCG CTG CTG GCC GCG CTG GCA CCC GCC CGC ACG GAC GGC GGG 1152
Ala His Ala Leu Leu Ala Ala Leu Ala Pro Ala Arg Thr Asp Gly Gly
370 375 380

10 GGC GGG GGC AGC ATC CCT GCA GCG CAA TCT GCA ACG GAA GCG AGG GGC 1200
Gly Gly Gly Ser Ile Pro Ala Ala Gln Ser Ala Thr Glu Ala Arg Gly
385 390 395 400

15 GCG GAG CCG ACT GCG GGC ATC CAC TGG TAC TCG CAG CTG CTC TAC CAC 1248
Ala Glu Pro Thr Ala Gly Ile His Trp Tyr Ser Gln Leu Leu Tyr His
405 410 415

ATT GGC ACC TGG CTG TTG GAC AGC GAG ACC ATG CAT CCC TTG GGA ATG 1296
Ile Gly Thr Trp Leu Leu Asp Ser Glu Thr Met His Pro Leu Gly Met
420 425 430

20 GCG GTC AAG TCC AGC TG 1313
Ala Val Lys Ser Ser
435

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1256 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..1257

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

45 ATG CGG CTT TTG ACG AGA GTG CTG CTG GTG TCT CTT CTC ACT CTG TCC 48
Met Arg Leu Leu Thr Arg Val Leu Leu Val Ser Leu Leu Thr Leu Ser
1 5 10 15

50 TTG GTG GTG TCC GGA CTG GCC TGC GGT CCT GGC AGA GGC TAC GGC AGA 96
Leu Val Val Ser Gly Leu Ala Cys Gly Pro Gly Arg Gly Tyr Gly Arg
20 25 30

55 AGA AGA CAT CCG AAG AAG CTG ACA CCT CTC GCC TAC AAG CAG TTC ATA 144
Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys Gln Phe Ile
35 40 45

CCT AAT GTC GCG GAG AAG ACC TTA GGG GCC AGC GGC AGA TAC GAG GGC 192
Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Arg Tyr Glu Gly
50 55 60

60 AAG ATA ACG CGC AAT TCG GAG AGA TTT AAA GAA CTT ACT CCA AAT TAC 240

- 69 -

Ser Gly Gln Leu Lys Ser Val Ile Val Gln Arg Ile Tyr Thr Glu Glu
 305 310 315 320

5 CAG CGG GGC TCG TTC GCA CCA GTG ACT GCA CAT GGG ACC ATT GTG GTC 1008
 Gln Arg Gly Ser Phe Ala Pro Val Thr Ala His Gly Thr Ile Val Val
 325 330 335

10 GAC AGA ATA CTG GCG TCC TGT TAC GCC GTA ATA GAG GAC CAG GGG CTT 1056
 Asp Arg Ile Leu Ala Ser Cys Tyr Ala Val Ile Glu Asp Gln Gly Leu
 340 345 350

15 GCG CAT TTG GCC TTC GCG CCC GCC AGG CTC TAT TAT TAC GTG TCA TCA 1104
 Ala His Leu Ala Phe Ala Pro Ala Arg Leu Tyr Tyr Tyr Val Ser Ser
 355 360 365

20 TTC CTG TCC CCC AAA ACT CCA GCA GTC GGT CCA ATG CGA CTT TAC AAC 1152
 Phe Leu Ser Pro Lys Thr Pro Ala Val Gly Pro Met Arg Leu Tyr Asn
 370 375 380

25 AGG AGG GGG TCC ACT GGT ACT CCA GGC TCC TGT CAT CAA ATG GGA ACG 1200
 Arg Arg Gly Ser Thr Gly Thr Pro Gly Ser Cys His Gln Met Gly Thr
 385 390 395 400

30 TGG CTT TTG GAC AGC AAC ATG CTT CAT CCT TTG GGG ATG TCA GTA AAC 1248
 Trp Leu Leu Asp Ser Asn Met Leu His Pro Leu Gly Met Ser Val Asn
 405 410 415

35 TCA AGC TG 1256
 Ser Ser

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1425 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 1..1425

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

50 ATG CTG CTG CTG GCG AGA TGT CTG CTG CTA GTC CTC GTC TCC TCG CTG 48
 Met Leu Leu Leu Ala Arg Cys Leu Leu Leu Val Leu Val Ser Ser Leu
 1 5 10 15

55 CTG GTA TGC TCG GGA CTG GCG TGC GGA CCG GGC AGG GGG TTC GGG AAG 96
 Leu Val Cys Ser Gly Leu Ala Cys Gly Pro Gly Arg Gly Phe Gly Lys
 20 25 30

60 AGG AGG CAC CCC AAA AAG CTG ACC CCT TTA GCC TAC AAG CAG TTT ATC 144
 Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys Gln Phe Ile

- 71 -

55

60

- 72 -

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 51..1283

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

10	CATCAGCCCA CCAGGAGACC TCGCCCGCCG CTCCCCCGGG CTCCCCGGCC ATG TCT	56
	Met Ser	
	1	
15	CCC GCC CGG CTC CGG CCC CGA CTG CAC TTC TGC CTG GTC CTG TTG CTG	104
	Pro Ala Arg Leu Arg Pro Arg Leu His Phe Cys Leu Val Leu Leu Leu	
	5 10 15	
20	CTG CTG GTG GTG CCC GCG GCA TGG GGC TGC GGG CCG GGT CGG GTG GTG	152
	Leu Leu Val Val Pro Ala Ala Trp Gly Cys Gly Pro Gly Arg Val Val	
	20 25 30	
25	GGC AGC CGC CGG CGA CCG CCA CGC AAA CTC GTG CCG CTC GCC TAC AAG	200
	Gly Ser Arg Arg Arg Pro Pro Arg Lys Leu Val Pro Leu Ala Tyr Lys	
	35 40 45 50	
30	CAG TTC AGC CCC AAT GTG CCC GAG AAG ACC CTG GGC GCC AGC GGA CGC	248
	Gln Phe Ser Pro Asn Val Pro Glu Lys Thr Leu Gly Ala Ser Gly Arg	
	55 60 65	
35	TAT GAA GGC AAG ATC GCT CGC AGC TCC GAG CGC TTC AAG GAG CTC ACC	296
	Tyr Glu Gly Lys Ile Ala Arg Ser Ser Glu Arg Phe Lys Glu Leu Thr	
	70 75 80	
40	CCC AAT TAC AAT CCA GAC ATC ATC TTC AAG GAC GAG GAG AAC ACA GGC	344
	Pro Asn Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Gly	
	85 90 95	
45	GCC GAC CGC CTC ATG ACC CAG CGC TGC AAG GAC CGC CTG AAC TCG CTG	392
	Ala Asp Arg Leu Met Thr Gln Arg Cys Lys Asp Arg Leu Asn Ser Leu	
	100 105 110	
50	GCT ATC TCG GTG ATG AAC CAG TGG CCC GGT GTG AAG CTG CGG GTG ACC	440
	Ala Ile Ser Val Met Asn Gln Trp Pro Gly Val Lys Leu Arg Val Thr	
	115 120 125 130	
55	GAG GGC TGG GAC GAG GAC GGC CAC CAC TCA GAG GAG TCC CTG CAT TAT	488
	Glu Gly Trp Asp Glu Asp Gly His His Ser Glu Glu Ser Leu His Tyr	
	135 140 145	
60	GAG GGC CGC GCG GTG GAC ATC ACC ACA TCA GAC CGC GAC CGC AAT AAG	536
	Glu Gly Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg Asn Lys	
	150 155 160	
65	TAT GGA CTG CTG GCG CGC TTG GCA GTG GAG GCC GGC TTT GAC TGG GTG	584
	Tyr Gly Leu Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val	
	165 170 175	
70	TAT TAC GAG TCA AAG GCC CAC GTG CAT TGC TCC GTC AAG TCC GAG CAC	632
	Tyr Tyr Glu Ser Lys Ala His Val His Cys Ser Val Lys Ser Glu His	

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AAGGGACCTG AGCTGGGGGA CACTGGCTCC TGCCATCTCC TCTGCCATGA AGATACACCA 1423
 TTGAGACTTG ACTGGGCAAC ACCAGCGTCC CCCACCCGCG TCGTGGTGTA GTCATAGAGC 1483
 5 TGCAAGCTGA GCTGGCGAGG GGATGGTTGT TGACCCCTCT CTCCTAGAGA CCTTGAGGCT 1543
 GGCACGGCGA CTCCCAACTC AGCCTGCTCT CACTACGAGT TTTCATACTC TGCCTCCCCC 1603
 ATTGGGAGGG CCCATTCCC 1622

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1191 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: both
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
 (B) LOCATION: 1..1191

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ATG GCT CTC CTG ACC AAT CTA CTG CCC TTG TGC TGC TTG GCA CTT CTG 48
 Met Ala Leu Leu Thr Asn Leu Leu Pro Leu Cys Cys Leu Ala Leu Leu
 1 5 10 15
 GCG CTG CCA GCC CAG AGC TGC GGG CCG GGC CGG GGG CCG GTT GGC CGG 96
 Ala Leu Pro Ala Gln Ser Cys Gly Pro Gly Arg Gly Pro Val Gly Arg
 20 25 30
 CGC CGC TAT GCG CGC AAG CAG CTC GTG CCG CTA CTC TAC AAG CAA TTT 144
 Arg Arg Tyr Ala Arg Lys Gln Leu Val Pro Leu Leu Tyr Lys Gln Phe
 35 40 45
 GTG CCC GGC GTG CCA GAG CGG ACC CTG GGC GCC AGT GGG CCA GCG GAG 192
 Val Pro Gly Val Pro Glu Arg Thr Leu Gly Ala Ser Gly Pro Ala Glu
 50 55 60
 GGG AGG GTG GCA AGG GGC TCC GAG CGC TTC CGG GAC CTC GTG CCC AAC 240
 Gly Arg Val Ala Arg Gly Ser Glu Arg Phe Arg Asp Leu Val Pro Asn
 65 70 75 80
 TAC AAC CCC GAC ATC ATC TTC AAG GAT GAG GAG AAC AGT GGA GCC GAC 288
 Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Ser Gly Ala Asp
 85 90 95
 CGC CTG ATG ACC GAG CGT TGC AAG GAG AGG GTG AAC GCT TTG GCC ATT 336
 Arg Leu Met Thr Glu Arg Cys Lys Glu Arg Val Asn Ala Leu Ala Ile
 100 105 110
 GCC GTG ATG AAC ATG TGG CCC GGA GTG CGC CTA CGA GTG ACT GAG GGC 384
 Ala Val Met Asn Met Trp Pro Gly Val Arg Leu Arg Val Thr Glu Gly
 115 120 125

60

CTG	CTC	CCC	GGC	GGG	GCC	GTC	CAG	CCG	ACT	GGC	ATG	CAT	TGG	TAC	TCT	1152
Leu	Leu	Pro	Gly	Gly	Ala	Val	Gln	Pro	Thr	Gly	Met	His	Trp	Tyr	Ser	
370						375					380					

5	CGG	CTC	CTC	TAC	CGC	TTA	GCG	GAG	GAG	CTA	CTG	GGC	TG			1191
	Arg	Leu	Leu	Tyr	Arg	Leu	Ala	Glu	Glu	Leu	Leu	Gly				
	385					390					395					

10

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

15

- (A) LENGTH: 1251 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

25

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..1248

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

30

ATG	GAC	GTA	AGG	CTG	CAT	CTG	AAG	CAA	TTT	GCT	TTA	CTG	TGT	TTT	ATC	48
Met	Asp	Val	Arg	Leu	His	Leu	Lys	Gln	Phe	Ala	Leu	Leu	Cys	Phe	Ile	
1				5				10						15		

35

AGC	TTG	CTT	CTG	ACG	CCT	TGT	GGA	TTA	GCC	TGT	GGT	CCT	GGT	AGA	GGT	96
Ser	Leu	Leu	Leu	Thr	Pro	Cys	Gly	Leu	Ala	Cys	Gly	Pro	Gly	Arg	Gly	
			20				25					30				

40

TAT	GGA	AAA	CGA	AGA	CAC	CCA	AAG	AAA	TTA	ACC	CCG	TTG	GCT	TAC	AAG	144
Tyr	Gly	Lys	Arg	Arg	His	Pro	Lys	Lys	Leu	Thr	Pro	Leu	Ala	Tyr	Lys	
		35					40				45					

45

CAA	TTC	ATC	CCC	AAC	GTT	GCT	GAG	AAA	ACG	CTT	GGA	GCC	AGC	GGC	AAA	192
Gln	Phe	Ile	Pro	Asn	Val	Ala	Glu	Lys	Thr	Leu	Gly	Ala	Ser	Gly	Lys	
	50					55					60					

50

TAC	GAA	GGC	AAA	ATC	ACA	AGG	AAT	TCA	GAG	AGA	TTT	AAA	GAG	CTG	ATT	240
Tyr	Glu	Gly	Lys	Ile	Thr	Arg	Asn	Ser	Glu	Arg	Phe	Lys	Glu	Leu	Ile	
	65				70				75					80		

55

CCG	AAT	TAT	AAT	CCC	GAT	ATC	ATC	TTT	AAG	GAC	GAG	GAA	AAC	ACA	AAC	288
Pro	Asn	Tyr	Asn	Pro	Asp	Ile	Ile	Phe	Lys	Asp	Glu	Glu	Asn	Thr	Asn	
			85					90					95			

60

GCT	GAC	AGG	CTG	ATG	ACC	AAG	CGC	TGT	AAG	GAC	AAG	TTA	AAT	TCG	TTG	336
Ala	Asp	Arg	Leu	Met	Thr	Lys	Arg	Cys	Lys	Asp	Lys	Leu	Asn	Ser	Leu	
			100				105					110				

GCC	ATA	TCC	GTC	ATG	AAC	CAC	TGG	CCC	GGC	GTG	AAA	CTG	CGC	GTC	ACT	384
Ala	Ile	Ser	Val	Met	Asn	His	Trp	Pro	Gly	Val	Lys	Leu	Arg	Val	Thr	
		115					120					125				

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	GAA	GGC	TGG	GAT	GAG	GAT	GGT	CAC	CAT	TTA	GAA	GAA	TCT	TTG	CAC	TAT	432
	Glu	Gly	Trp	Asp	Glu	Asp	Gly	His	His	Leu	Glu	Glu	Ser	Leu	His	Tyr	
		130					135					140					
5	GAG	GGA	CGG	GCA	GTG	GAC	ATC	ACT	ACC	TCA	GAC	AGG	GAT	AAA	AGC	AAG	480
	Glu	Gly	Arg	Ala	Val	Asp	Ile	Thr	Thr	Ser	Asp	Arg	Asp	Lys	Ser	Lys	
		145				150					155					160	
10	TAT	GGG	ATG	CTA	TCC	AGG	CTT	GCA	GTG	GAG	GCA	GGA	TTC	GAC	TGG	GTC	528
	Tyr	Gly	Met	Leu	Ser	Arg	Leu	Ala	Val	Glu	Ala	Gly	Phe	Asp	Trp	Val	
					165					170					175		
15	TAT	TAT	GAA	TCT	AAA	GCC	CAC	ATA	CAC	TGC	TCT	GTC	AAA	GCA	GAA	AAT	576
	Tyr	Tyr	Glu	Ser	Lys	Ala	His	Ile	His	Cys	Ser	Val	Lys	Ala	Glu	Asn	
				180					185					190			
20	TCA	GTG	GCT	GCT	AAA	TCA	GGA	GGA	TGT	TTT	CCT	GGG	TCT	GGG	ACG	GTG	624
	Ser	Val	Ala	Ala	Lys	Ser	Gly	Gly	Cys	Phe	Pro	Gly	Ser	Gly	Thr	Val	
			195					200					205				
25	ACA	CTT	GGT	GAT	GGG	ACG	AGG	AAA	CCC	ATC	AAA	GAT	CTT	AAA	GTG	GGC	672
	Thr	Leu	Gly	Asp	Gly	Thr	Arg	Lys	Pro	Ile	Lys	Asp	Leu	Lys	Val	Gly	
		210					215					220					
30	GAC	CGG	GTT	TTG	GCT	GCA	GAC	GAG	AAG	GGA	AAT	GTC	TTA	ATA	AGC	GAC	720
	Asp	Arg	Val	Leu	Ala	Ala	Asp	Glu	Lys	Gly	Asn	Val	Leu	Ile	Ser	Asp	
		225				230					235					240	
35	TTT	ATT	ATG	TTT	ATA	GAC	CAC	GAT	CCG	ACA	ACG	AGA	AGG	CAA	TTC	ATC	768
	Phe	Ile	Met	Phe	Ile	Asp	His	Asp	Pro	Thr	Thr	Arg	Arg	Gln	Phe	Ile	
					245					250					255		
40	GTC	ATC	GAG	ACG	TCA	GAA	CCT	TTC	ACC	AAG	CTC	ACC	CTC	ACT	GCC	GCG	816
	Val	Ile	Glu	Thr	Ser	Glu	Pro	Phe	Thr	Lys	Leu	Thr	Leu	Thr	Ala	Ala	
				260					265					270			
45	CAC	CTA	GTT	TTC	GTT	GGA	AAC	TCT	TCA	GCA	GCT	TCG	GGT	ATA	ACA	GCA	864
	His	Leu	Val	Phe	Val	Gly	Asn	Ser	Ser	Ala	Ala	Ser	Gly	Ile	Thr	Ala	
			275					280					285				
50	ACA	TTT	GCC	AGC	AAC	GTG	AAG	CCT	GGA	GAT	ACA	GTT	TTA	GTG	TGG	GAA	912
	Thr	Phe	Ala	Ser	Asn	Val	Lys	Pro	Gly	Asp	Thr	Val	Leu	Val	Trp	Glu	
		290					295					300					
55	GAC	ACA	TGC	GAG	AGC	CTC	AAG	AGC	GTT	ACA	GTG	AAA	AGG	ATT	TAC	ACT	960
	Asp	Thr	Cys	Glu	Ser	Leu	Lys	Ser	Val	Thr	Val	Lys	Arg	Ile	Tyr	Thr	
		305				310					315					320	
60	GAG	GAG	CAC	GAG	GGC	TCT	TTT	GCG	CCA	GTC	ACC	GCG	CAC	GGA	ACC	ATA	1008
	Glu	Glu	His	Glu	Gly	Ser	Phe	Ala	Pro	Val	Thr	Ala	His	Gly	Thr	Ile	
					325					330					335		
65	ATA	GTG	GAT	CAG	GTG	TTG	GCA	TCG	TGC	TAC	GCG	GTC	ATT	GAG	AAC	CAC	1056
	Ile	Val	Asp	Gln	Val	Leu	Ala	Ser	Cys	Tyr	Ala	Val	Ile	Glu	Asn	His	
				340					345					350			
70	AAA	TGG	GCA	CAT	TGG	GCT	TTT	GCG	CCG	GTC	AGG	TTG	TGT	CAC	AAG	CTG	1104
	Lys	Trp	Ala	His	Trp	Ala	Phe	Ala	Pro	Val	Arg	Leu	Cys	His	Lys	Leu	
			355					360					365				

ATG ACG TGG CTT TTT CCG GCT CGT GAA TCA AAC GTC AAT TTT CAG GAG 1152
Met Thr Trp Leu Phe Pro Ala Arg Glu Ser Asn Val Asn Phe Gln Glu
370 375 380

5 GAT GGT ATC CAC TGG TAC TCA AAT ATG CTG TTT CAC ATC GGC TCT TGG 1200
Asp Gly Ile His Trp Tyr Ser Asn Met Leu Phe His Ile Gly Ser Trp
385 390 395 400

10 CTG CTG GAC AGA GAC TCT TTC CAT CCA CTC GGG ATT TTA CAC TTA AGT 1248
Leu Leu Asp Arg Asp Ser Phe His Pro Leu Gly Ile Leu His Leu Ser
405 410 415

TGA 1251

15

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

20

- (A) LENGTH: 425 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Met Val Glu Met Leu Leu Leu Thr Arg Ile Leu Leu Val Gly Phe Ile
1 5 10 15

30

Cys Ala Leu Leu Val Ser Ser Gly Leu Thr Cys Gly Pro Gly Arg Gly
20 25 30

35

Ile Gly Lys Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys
35 40 45

Gln Phe Ile Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Arg
50 55 60

40

Tyr Glu Gly Lys Ile Thr Arg Asn Ser Glu Arg Phe Lys Glu Leu Thr
65 70 75 80

Pro Asn Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Gly
85 90 95

45

Ala Asp Arg Leu Met Thr Gln Arg Cys Lys Asp Lys Leu Asn Ala Leu
100 105 110

Ala Ile Ser Val Met Asn Gln Trp Pro Gly Val Lys Leu Arg Val Thr
115 120 125

50

Glu Gly Trp Asp Glu Asp Gly His His Ser Glu Glu Ser Leu His Tyr
130 135 140

55

Glu Gly Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg Ser Lys
145 150 155 160

Tyr Gly Met Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val
165 170 175

60

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Tyr Tyr Glu Ser Lys Ala His Ile His Cys Ser Val Lys Ala Glu Asn
 180 185 190
 5 Ser Val Ala Ala Lys Ser Gly Gly Cys Phe Pro Gly Ser Ala Thr Val
 195 200 205
 His Leu Glu His Gly Gly Thr Lys Leu Val Lys Asp Leu Ser Pro Gly
 210 215 220
 10 Asp Arg Val Leu Ala Ala Asp Ala Asp Gly Arg Leu Leu Tyr Ser Asp
 225 230 235 240
 Phe Leu Thr Phe Leu Asp Arg Met Asp Ser Ser Arg Lys Leu Phe Tyr
 245 250 255
 15 Val Ile Glu Thr Arg Gln Pro Arg Ala Arg Leu Leu Leu Thr Ala Ala
 260 265 270
 His Leu Leu Phe Val Ala Pro Gln His Asn Gln Ser Glu Ala Thr Gly
 275 280 285
 20 Ser Thr Ser Gly Gln Ala Leu Phe Ala Ser Asn Val Lys Pro Gly Gln
 290 295 300
 25 Arg Val Tyr Val Leu Gly Glu Gly Gly Gln Gln Leu Leu Pro Ala Ser
 305 310 315 320
 Val His Ser Val Ser Leu Arg Glu Glu Ala Ser Gly Ala Tyr Ala Pro
 325 330 335
 30 Leu Thr Ala Gln Gly Thr Ile Leu Ile Asn Arg Val Leu Ala Ser Cys
 340 345 350
 Tyr Ala Val Ile Glu Glu His Ser Trp Ala His Trp Ala Phe Ala Pro
 355 360 365
 35 Phe Arg Leu Ala Gln Gly Leu Leu Ala Ala Leu Cys Pro Asp Gly Ala
 370 375 380
 40 Ile Pro Thr Ala Ala Thr Thr Thr Thr Gly Ile His Trp Tyr Ser Arg
 385 390 395 400
 Leu Leu Tyr Arg Ile Gly Ser Trp Val Leu Asp Gly Asp Ala Leu His
 405 410 415
 45 Pro Leu Gly Met Val Ala Pro Ala Ser
 420 425

50 (2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 396 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

60

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

5	Met	Ala	Leu	Pro	Ala	Ser	Leu	Leu	Pro	Leu	Cys	Cys	Leu	Ala	Leu	Leu	1	5	10	15
	Ala	Leu	Ser	Ala	Gln	Ser	Cys	Gly	Pro	Gly	Arg	Gly	Pro	Val	Gly	Arg	20	25	30	
10	Arg	Arg	Tyr	Val	Arg	Lys	Gln	Leu	Val	Pro	Leu	Leu	Tyr	Lys	Gln	Phe	35	40	45	
	Val	Pro	Ser	Met	Pro	Glu	Arg	Thr	Leu	Gly	Ala	Ser	Gly	Pro	Ala	Glu	50	55	60	
15	Gly	Arg	Val	Thr	Arg	Gly	Ser	Glu	Arg	Phe	Arg	Asp	Leu	Val	Pro	Asn	65	70	75	80
	Tyr	Asn	Pro	Asp	Ile	Ile	Phe	Lys	Asp	Glu	Glu	Asn	Ser	Gly	Ala	Asp	85	90	95	
20	Arg	Leu	Met	Thr	Glu	Arg	Cys	Lys	Glu	Arg	Val	Asn	Ala	Leu	Ala	Ile	100	105	110	
25	Ala	Val	Met	Asn	Met	Trp	Pro	Gly	Val	Arg	Leu	Arg	Val	Thr	Glu	Gly	115	120	125	
	Trp	Asp	Glu	Asp	Gly	His	His	Ala	Gln	Asp	Ser	Leu	His	Tyr	Glu	Gly	130	135	140	
30	Arg	Ala	Leu	Asp	Ile	Thr	Thr	Ser	Asp	Arg	Asp	Arg	Asn	Lys	Tyr	Gly	145	150	155	160
	Leu	Leu	Ala	Arg	Leu	Ala	Val	Glu	Ala	Gly	Phe	Asp	Trp	Val	Tyr	Tyr	165	170	175	
35	Glu	Ser	Arg	Asn	His	Ile	His	Val	Ser	Val	Lys	Ala	Asp	Asn	Ser	Leu	180	185	190	
40	Ala	Val	Arg	Ala	Gly	Gly	Cys	Phe	Pro	Gly	Asn	Ala	Thr	Val	Arg	Leu	195	200	205	
	Arg	Ser	Gly	Glu	Arg	Lys	Gly	Leu	Arg	Glu	Leu	His	Arg	Gly	Asp	Trp	210	215	220	
45	Val	Leu	Ala	Ala	Asp	Ala	Ala	Gly	Arg	Val	Val	Pro	Thr	Pro	Val	Leu	225	230	235	240
	Leu	Phe	Leu	Asp	Arg	Asp	Leu	Gln	Arg	Arg	Ala	Ser	Phe	Val	Ala	Val	245	250	255	
50	Glu	Thr	Glu	Arg	Pro	Pro	Arg	Lys	Leu	Leu	Leu	Thr	Pro	Trp	His	Leu	260	265	270	
55	Val	Phe	Ala	Ala	Arg	Gly	Pro	Ala	Pro	Ala	Pro	Gly	Asp	Phe	Ala	Pro	275	280	285	
	Val	Phe	Ala	Arg	Arg	Leu	Arg	Ala	Gly	Asp	Ser	Val	Leu	Ala	Pro	Gly	290	295	300	
60	Gly	Asp	Ala	Leu	Gln	Pro	Ala	Arg	Val	Ala	Arg	Val	Ala	Arg	Glu	Glu				

305 310 315 320
 Ala Val Gly Val Phe Ala Pro Leu Thr Ala His Gly Thr Leu Leu Val
 325 330 335
 5 Asn Asp Val Leu Ala Ser Cys Tyr Ala Val Leu Glu Ser His Gln Trp
 340 345 350
 10 Ala His Arg Ala Phe Ala Pro Leu Arg Leu Leu His Ala Leu Gly Ala
 355 360 365
 Leu Leu Pro Gly Gly Ala Val Gln Pro Thr Gly Met His Trp Tyr Ser
 370 375 380
 15 Arg Leu Leu Tyr Arg Leu Ala Glu Glu Leu Met Gly
 385 390 395

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 411 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Met Ser Pro Ala Trp Leu Arg Pro Arg Leu Arg Phe Cys Leu Phe Leu
 1 5 10 15
 35 Leu Leu Leu Leu Leu Val Pro Ala Ala Arg Gly Cys Gly Pro Gly Arg
 20 25 30
 Val Val Gly Ser Arg Arg Arg Pro Pro Arg Lys Leu Val Pro Leu Ala
 35 40 45
 Tyr Lys Gln Phe Ser Pro Asn Val Pro Glu Lys Thr Leu Gly Ala Ser
 50 55 60
 45 Gly Arg Tyr Glu Gly Lys Ile Ala Arg Ser Ser Glu Arg Phe Lys Glu
 65 70 75 80
 Leu Thr Pro Asn Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn
 85 90 95
 50 Thr Gly Ala Asp Arg Leu Met Thr Gln Arg Cys Lys Asp Arg Leu Asn
 100 105 110
 Ser Leu Ala Ile Ser Val Met Asn Gln Trp Pro Gly Val Lys Leu Arg
 115 120 125
 55 Val Thr Glu Gly Arg Asp Glu Asp Gly His His Ser Glu Glu Ser Leu
 130 135 140
 60 His Tyr Glu Gly Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg
 145 150 155 160

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Asn Lys Tyr Gly Leu Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp
165 170 175

5 Trp Val Tyr Tyr Glu Ser Lys Ala His Val His Cys Ser Val Lys Ser
180 185 190

Glu His Ser Ala Ala Ala Lys Thr Gly Gly Cys Phe Pro Ala Gly Ala
195 200 205

10 Gln Val Arg Leu Glu Asn Gly Glu Arg Val Ala Leu Ser Ala Val Lys
210 215 220

15 Pro Gly Asp Arg Val Leu Ala Met Gly Glu Asp Gly Thr Pro Thr Phe
225 230 235 240

Ser Asp Val Leu Ile Phe Leu Asp Arg Glu Pro Asn Arg Leu Arg Ala
245 250 255

20 Phe Gln Val Ile Glu Thr Gln Asp Pro Pro Arg Arg Leu Ala Leu Thr
260 265 270

Pro Ala His Leu Leu Phe Ile Ala Asp Asn His Thr Glu Pro Ala Ala
275 280 285

25 His Phe Arg Ala Thr Phe Ala Ser His Val Gln Pro Gly Gln Tyr Val
290 295 300

30 Leu Val Ser Gly Val Pro Gly Leu Gln Pro Ala Arg Val Ala Ala Val
305 310 315 320

Ser Thr His Val Ala Leu Gly Ser Tyr Ala Pro Leu Thr Arg His Gly
325 330 335

35 Thr Leu Val Val Glu Asp Val Val Ala Ser Cys Phe Ala Ala Val Ala
340 345 350

Asp His His Leu Ala Gln Leu Ala Phe Trp Pro Leu Arg Leu Phe Pro
355 360 365

40 Ser Leu Ala Trp Gly Ser Trp Thr Pro Ser Glu Gly Val His Ser Tyr
370 375 380

45 Pro Gln Met Leu Tyr Arg Leu Gly Arg Leu Leu Leu Glu Glu Ser Thr
385 390 395 400

Phe His Pro Leu Gly Met Ser Gly Ala Gly Ser
405 410

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 437 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

5	Met	Leu	Leu	Leu	Leu	Ala	Arg	Cys	Phe	Leu	Val	Ile	Leu	Ala	Ser	Ser	
	1				5					10					15		
	Leu	Leu	Val	Cys	Pro	Gly	Leu	Ala	Cys	Gly	Pro	Gly	Arg	Gly	Phe	Gly	
				20					25					30			
10	Lys	Arg	Arg	His	Pro	Lys	Lys	Leu	Thr	Pro	Leu	Ala	Tyr	Lys	Gln	Phe	
				35				40					45				
	Ile	Pro	Asn	Val	Ala	Glu	Lys	Thr	Leu	Gly	Ala	Ser	Gly	Arg	Tyr	Glu	
15		50					55					60					
	Gly	Lys	Ile	Thr	Arg	Asn	Ser	Glu	Arg	Phe	Lys	Glu	Leu	Thr	Pro	Asn	
	65					70					75					80	
20	Tyr	Asn	Pro	Asp	Ile	Ile	Phe	Lys	Asp	Glu	Glu	Asn	Thr	Gly	Ala	Asp	
					85					90					95		
	Arg	Leu	Met	Thr	Gln	Arg	Cys	Lys	Asp	Lys	Leu	Asn	Ala	Leu	Ala	Ile	
				100					105					110			
25	Ser	Val	Met	Asn	Gln	Trp	Pro	Gly	Val	Arg	Leu	Arg	Val	Thr	Glu	Gly	
			115					120					125				
	Trp	Asp	Glu	Asp	Gly	His	His	Ser	Glu	Glu	Ser	Leu	His	Tyr	Glu	Gly	
30		130					135					140					
	Arg	Ala	Val	Asp	Ile	Thr	Thr	Ser	Asp	Arg	Asp	Arg	Ser	Lys	Tyr	Gly	
	145					150				155						160	
35	Met	Leu	Ala	Arg	Leu	Ala	Val	Glu	Ala	Gly	Phe	Asp	Trp	Val	Tyr	Tyr	
					165					170					175		
	Glu	Ser	Lys	Ala	His	Ile	His	Cys	Ser	Val	Lys	Ala	Glu	Asn	Ser	Val	
				180					185					190			
40	Ala	Ala	Lys	Ser	Gly	Gly	Cys	Phe	Pro	Gly	Ser	Ala	Thr	Val	His	Leu	
			195					200					205				
	Glu	Gln	Gly	Gly	Thr	Lys	Leu	Val	Lys	Asp	Leu	Arg	Pro	Gly	Asp	Arg	
45		210					215					220					
	Val	Leu	Ala	Ala	Asp	Asp	Gln	Gly	Arg	Leu	Leu	Tyr	Ser	Asp	Phe	Leu	
	225					230					235					240	
50	Thr	Phe	Leu	Asp	Arg	Asp	Glu	Gly	Ala	Lys	Lys	Val	Phe	Tyr	Val	Ile	
				245						250					255		
	Glu	Thr	Leu	Glu	Pro	Arg	Glu	Arg	Leu	Leu	Leu	Thr	Ala	Ala	His	Leu	
				260				265					270				
55	Leu	Phe	Val	Ala	Pro	His	Asn	Asp	Ser	Gly	Pro	Thr	Pro	Gly	Pro	Ser	
			275					280					285				
	Ala	Leu	Phe	Ala	Ser	Arg	Val	Arg	Pro	Gly	Gln	Arg	Val	Tyr	Val	Val	
60		290					295					300					

Ala Glu Arg Gly Gly Asp Arg Arg Leu Leu Pro Ala Ala Val His Ser
305 310 315 320

5 Val Thr Leu Arg Glu Glu Glu Ala Gly Ala Tyr Ala Pro Leu Thr Ala
325 330 335

His Gly Thr Ile Leu Ile Asn Arg Val Leu Ala Ser Cys Tyr Ala Val
340 345 350

10 Ile Glu Glu His Ser Trp Ala His Arg Ala Phe Ala Pro Phe Arg Leu
355 360 365

15 Ala His Ala Leu Leu Ala Ala Leu Ala Pro Ala Arg Thr Asp Gly Gly
370 375 380

Gly Gly Gly Ser Ile Pro Ala Ala Gln Ser Ala Thr Glu Ala Arg Gly
385 390 395 400

20 Ala Glu Pro Thr Ala Gly Ile His Trp Tyr Ser Gln Leu Leu Tyr His
405 410 415

Ile Gly Thr Trp Leu Leu Asp Ser Glu Thr Met His Pro Leu Gly Met
420 425 430

25 Ala Val Lys Ser Ser
435

30 (2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 418 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Met Arg Leu Leu Thr Arg Val Leu Leu Val Ser Leu Leu Thr Leu Ser
1 5 10 15

Leu Val Val Ser Gly Leu Ala Cys Gly Pro Gly Arg Gly Tyr Gly Arg
20 25 30

Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys Gln Phe Ile
35 40 45

Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Arg Tyr Glu Gly
50 55 60

Lys Ile Thr Arg Asn Ser Glu Arg Phe Lys Glu Leu Thr Pro Asn Tyr
65 70 75 80

Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Gly Ala Asp Arg
85 90 95

5 Leu Met Thr Gln Arg Cys Lys Asp Lys Leu Asn Ser Leu Ala Ile Ser
 100 105 110
 Val Met Asn His Trp Pro Gly Val Lys Leu Arg Val Thr Glu Gly Trp
 115 120 125
 Asp Glu Asp Gly His His Phe Glu Glu Ser Leu His Tyr Glu Gly Arg
 130 135 140
 10 Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Lys Ser Lys Tyr Gly Thr
 145 150 155 160
 Leu Ser Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr Glu
 165 170 175
 15 Ser Lys Ala His Ile His Cys Ser Val Lys Ala Glu Asn Ser Val Ala
 180 185 190
 20 Ala Lys Ser Gly Gly Cys Phe Pro Gly Ser Ala Leu Val Ser Leu Gln
 195 200 205
 Asp Gly Gly Gln Lys Ala Val Lys Asp Leu Asn Pro Gly Asp Lys Val
 210 215 220
 25 Leu Ala Ala Asp Ser Ala Gly Asn Leu Val Phe Ser Asp Phe Ile Met
 225 230 235 240
 Phe Thr Asp Arg Asp Ser Thr Thr Arg Arg Val Phe Tyr Val Ile Glu
 245 250 255
 30 Thr Gln Glu Pro Val Glu Lys Ile Thr Leu Thr Ala Ala His Leu Leu
 260 265 270
 Phe Val Leu Asp Asn Ser Thr Glu Asp Leu His Thr Met Thr Ala Ala
 275 280 285
 35 Tyr Ala Ser Ser Val Arg Ala Gly Gln Lys Val Met Val Val Asp Asp
 290 295 300
 40 Ser Gly Gln Leu Lys Ser Val Ile Val Gln Arg Ile Tyr Thr Glu Glu
 305 310 315 320
 Gln Arg Gly Ser Phe Ala Pro Val Thr Ala His Gly Thr Ile Val Val
 325 330 335
 45 Asp Arg Ile Leu Ala Ser Cys Tyr Ala Val Ile Glu Asp Gln Gly Leu
 340 345 350
 50 Ala His Leu Ala Phe Ala Pro Ala Arg Leu Tyr Tyr Tyr Val Ser Ser
 355 360 365
 Phe Leu Ser Pro Lys Thr Pro Ala Val Gly Pro Met Arg Leu Tyr Asn
 370 375 380
 55 Arg Arg Gly Ser Thr Gly Thr Pro Gly Ser Cys His Gln Met Gly Thr
 385 390 395 400
 Trp Leu Leu Asp Ser Asn Met Leu His Pro Leu Gly Met Ser Val Asn
 405 410 415
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Ser Ser

5 (2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 475 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Met Leu Leu Leu Ala Arg Cys Leu Leu Leu Val Leu Val Ser Ser Leu
1 5 10 15
Leu Val Cys Ser Gly Leu Ala Cys Gly Pro Gly Arg Gly Phe Gly Lys
20 25 30
Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys Gln Phe Ile
25 35 40 45
Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Arg Tyr Glu Gly
50 55 60
Lys Ile Ser Arg Asn Ser Glu Arg Phe Lys Glu Leu Thr Pro Asn Tyr
30 65 70 75 80
Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Gly Ala Asp Arg
35 85 90 95
Leu Met Thr Gln Arg Cys Lys Asp Lys Leu Asn Ala Leu Ala Ile Ser
100 105 110
Val Met Asn Gln Trp Pro Gly Val Lys Leu Arg Val Thr Glu Gly Trp
40 115 120 125
Asp Glu Asp Gly His His Ser Glu Glu Ser Leu His Tyr Glu Gly Arg
130 135 140
Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg Ser Lys Tyr Gly Met
45 145 150 155 160
Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr Glu
165 170 175
Ser Lys Ala His Ile His Cys Ser Val Lys Ala Glu Asn Ser Val Ala
50 180 185 190
Ala Lys Ser Gly Gly Cys Phe Pro Gly Ser Ala Thr Val His Leu Glu
55 195 200 205
Gln Gly Gly Thr Lys Leu Val Lys Asp Leu Ser Pro Gly Asp Arg Val
210 215 220
Leu Ala Ala Asp Asp Gln Gly Arg Leu Leu Tyr Ser Asp Phe Leu Thr
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225 230 235 240

Phe Leu Asp Arg Asp Asp Gly Ala Lys Lys Val Phe Tyr Val Ile Glu
 245 250 255

5 Thr Arg Glu Pro Arg Glu Arg Leu Leu Leu Thr Ala Ala His Leu Leu
 260 265 270

10 Phe Val Ala Pro His Asn Asp Ser Ala Thr Gly Glu Pro Glu Ala Ser
 275 280 285

Ser Gly Ser Gly Pro Pro Ser Gly Gly Ala Leu Gly Pro Arg Ala Leu
 290 295 300

15 Phe Ala Ser Arg Val Arg Pro Gly Gln Arg Val Tyr Val Val Ala Glu
305 310 315 320

Arg Asp Gly Asp Arg Arg Leu Leu Pro Ala Ala Val His Ser Val Thr
 325 330 335

20 Leu Ser Glu Glu Ala Ala Gly Ala Tyr Ala Pro Leu Thr Ala Gln Gly
 340 345 350

25 Thr Ile Leu Ile Asn Arg Val Leu Ala Ser Cys Tyr Ala Val Ile Glu
355 360 365

Glu His Ser Trp Ala His Arg Ala Phe Ala Pro Phe Arg Leu Ala His
370 375 380

30 Ala Leu Leu Ala Ala Leu Ala Pro Ala Arg Thr Asp Arg Gly Gly Asp
385 390 395 400

Ser Gly Gly Gly Asp Arg Gly Gly Gly Gly Gly Arg Val Ala Leu Thr
 405 410 415

35 Ala Pro Gly Ala Ala Asp Ala Pro Gly Ala Gly Ala Thr Ala Gly Ile
 420 425 430

40 His Trp Tyr Ser Gln Leu Leu Tyr Gln Ile Gly Thr Trp Leu Leu Asp
435 440 445

Ser Glu Ala Leu His Pro Leu Gly Met Ala Val Lys Ser Ser Xaa Ser
450 455 460

45 Arg Gly Ala Gly Gly Gly Ala Arg Glu Gly Ala
465 470 475

(2) INFORMATION FOR SEQ ID NO:16:

50 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 411 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

55 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

60 Met Ser Pro Ala Arg Leu Arg Pro Arg Leu His Phe Cys Leu Val Leu
 1 5 10 15

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Thr Leu Val Val Glu Asp Val Val Ala Ser Cys Phe Ala Ala Val Ala
340 345 350

5 Asp His His Leu Ala Gln Leu Ala Phe Trp Pro Leu Arg Leu Phe His
355 360 365

10 Ser Leu Ala Trp Gly Ser Trp Thr Pro Gly Glu Gly Val His Trp Tyr
370 375 380

Pro Gln Leu Leu Tyr Arg Leu Gly Arg Leu Leu Leu Glu Glu Gly Ser
385 390 395 400

15 Phe His Pro Leu Gly Met Ser Gly Ala Gly Ser
405 410

(2) INFORMATION FOR SEQ ID NO:17:

20 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 396 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

25 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Met Ala Leu Leu Thr Asn Leu Leu Pro Leu Cys Cys Leu Ala Leu Leu
1 5 10 15

Ala Leu Pro Ala Gln Ser Cys Gly Pro Gly Arg Gly Pro Val Gly Arg
20 25 30

Arg Arg Tyr Ala Arg Lys Gln Leu Val Pro Leu Leu Tyr Lys Gln Phe
35 40 45

Val Pro Gly Val Pro Glu Arg Thr Leu Gly Ala Ser Gly Pro Ala Glu
50 55 60

Gly Arg Val Ala Arg Gly Ser Glu Arg Phe Arg Asp Leu Val Pro Asn
65 70 75 80

Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Ser Gly Ala Asp
85 90 95

Arg Leu Met Thr Glu Arg Cys Lys Glu Arg Val Asn Ala Leu Ala Ile
100 105 110

Ala Val Met Asn Met Trp Pro Gly Val Arg Leu Arg Val Thr Glu Gly
115 120 125

Trp Asp Glu Asp Gly His His Ala Gln Asp Ser Leu His Tyr Glu Gly
130 135 140

Arg Ala Leu Asp Ile Thr Thr Ser Asp Arg Asp Arg Asn Lys Tyr Gly
145 150 155 160

Leu Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr
165 170 175

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(2) INFORMATION FOR SEQ ID NO:18:

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(ii) MOLECULE TYPE: protein

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Met Asp Val Arg Leu His Leu Lys Gln Phe Ala Leu Leu Cys Phe Ile
1 5 10 15

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Tyr Gly Lys Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys
 35 40 45
 5 Gln Phe Ile Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Lys
 50 55 60
 Tyr Glu Gly Lys Ile Thr Arg Asn Ser Glu Arg Phe Lys Glu Leu Ile
 65 70 75 80
 10 Pro Asn Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Asn
 85 90 95
 15 Ala Asp Arg Leu Met Thr Lys Arg Cys Lys Asp Lys Leu Asn Ser Leu
 100 105 110
 Ala Ile Ser Val Met Asn His Trp Pro Gly Val Lys Leu Arg Val Thr
 115 120 125
 20 Glu Gly Trp Asp Glu Asp Gly His His Leu Glu Glu Ser Leu His Tyr
 130 135 140
 Glu Gly Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Lys Ser Lys
 145 150 155 160
 25 Tyr Gly Met Leu Ser Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val
 165 170 175
 Tyr Tyr Glu Ser Lys Ala His Ile His Cys Ser Val Lys Ala Glu Asn
 180 185 190
 Ser Val Ala Ala Lys Ser Gly Gly Cys Phe Pro Gly Ser Gly Thr Val
 195 200 205
 35 Thr Leu Gly Asp Gly Thr Arg Lys Pro Ile Lys Asp Leu Lys Val Gly
 210 215 220
 Asp Arg Val Leu Ala Ala Asp Glu Lys Gly Asn Val Leu Ile Ser Asp
 225 230 235 240
 40 Phe Ile Met Phe Ile Asp His Asp Pro Thr Thr Arg Arg Gln Phe Ile
 245 250 255
 Val Ile Glu Thr Ser Glu Pro Phe Thr Lys Leu Thr Leu Thr Ala Ala
 260 265 270
 45 His Leu Val Phe Val Gly Asn Ser Ser Ala Ala Ser Gly Ile Thr Ala
 275 280 285
 50 Thr Phe Ala Ser Asn Val Lys Pro Gly Asp Thr Val Leu Val Trp Glu
 290 295 300
 Asp Thr Cys Glu Ser Leu Lys Ser Val Thr Val Lys Arg Ile Tyr Thr
 305 310 315 320
 55 Glu Glu His Glu Gly Ser Phe Ala Pro Val Thr Ala His Gly Thr Ile
 325 330 335
 Ile Val Asp Gln Val Leu Ala Ser Cys Tyr Ala Val Ile Glu Asn His
 340 345 350

Lys Trp Ala His Trp Ala Phe Ala Pro Val Arg Leu Cys His Lys Leu
355 360 365

5 Met Thr Trp Leu Phe Pro Ala Arg Glu Ser Asn Val Asn Phe Gln Glu
370 375 380

10 Asp Gly Ile His Trp Tyr Ser Asn Met Leu Phe His Ile Gly Ser Trp
385 390 395 400

Leu Leu Asp Arg Asp Ser Phe His Pro Leu Gly Ile Leu His Leu Ser
405 410 415

15 (2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1416 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

- (ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1413

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

ATG GAT AAC CAC AGC TCA GTG CCT TGG GCC AGT GCC GCC AGT GTC ACC 48
Met Asp Asn His Ser Ser Val Pro Trp Ala Ser Ala Ala Ser Val Thr
1 5 10 15

TGT CTC TCC CTG GGA TGC CAA ATG CCA CAG TTC CAG TTC CAG TTC CAG 96
Cys Leu Ser Leu Gly Cys Gln Met Pro Gln Phe Gln Phe Gln Phe Gln
20 25 30

CTC CAA ATC CGC AGC GAG CTC CAT CTC CGC AAG CCC GCA AGA AGA ACG 144
Leu Gln Ile Arg Ser Glu Leu His Leu Arg Lys Pro Ala Arg Arg Thr
35 40 45

CAA ACG ATG CGC CAC ATT GCG CAT ACG CAG CGT TGC CTC AGC AGG CTG 192
Gln Thr Met Arg His Ile Ala His Thr Gln Arg Cys Leu Ser Arg Leu
50 55 60

ACC TCT CTG GTG GCC CTG CTG CTG ATC GTC TTG CCG ATG GTC TTT AGC 240
Thr Ser Leu Val Ala Leu Leu Leu Ile Val Leu Pro Met Val Phe Ser
65 70 75 80

CCG GCT CAC AGC TGC GGT CCT GGC CGA GGA TTG GGT CGT CAT AGG GCG 288
Pro Ala His Ser Cys Gly Pro Gly Arg Gly Leu Gly Arg His Arg Ala
85 90 95

CGC AAC CTG TAT CCG CTG GTC CTC AAG CAG ACA ATT CCC AAT CTA TCC 336
Arg Asn Leu Tyr Pro Leu Val Leu Lys Gln Thr Ile Pro Asn Leu Ser
100 105 110

60

AAC CAG GTG CTC GTA CGG GAT GTG GAG ACG GGC GAG CTG AGG CCC CAG 1104
Asn Gln Val Leu Val Arg Asp Val Glu Thr Gly Glu Leu Arg Pro Gln
355 360 365

5 CGA GTG GTC AAG TTG GGC AGT GTG CGC AGT AAG GGC GTG GTC GCG CCG 1152
Arg Val Val Lys Leu Gly Ser Val Arg Ser Lys Gly Val Val Ala Pro
370 375 380

10 CTG ACC CGC GAG GGC ACC ATT GTG GTC AAC TCG GTG GCC GCC AGT TGC 1200
Leu Thr Arg Glu Gly Thr Ile Val Val Asn Ser Val Ala Ala Ser Cys
385 390 395 400

15 TAT GCG GTG ATC AAC AGT CAG TCG CTG GCC CAC TGG GGA CTG GCT CCC 1248
Tyr Ala Val Ile Asn Ser Gln Ser Leu Ala His Trp Gly Leu Ala Pro
405 410 415

20 ATG CGC CTG CTG TCC ACG CTG GAG GCG TGG CTG CCC GCC AAG GAG CAG 1296
Met Arg Leu Leu Ser Thr Leu Glu Ala Trp Leu Pro Ala Lys Glu Gln
420 425 430

25 TTG CAC AGT TCG CCG AAG GTG GTG AGC TCG GCG CAG CAG CAG AAT GGC 1344
Leu His Ser Ser Pro Lys Val Val Ser Ser Ala Gln Gln Gln Asn Gly
435 440 445

30 ATC CAT TGG TAT GCC AAT GCG CTC TAC AAG GTC AAG GAC TAC GTG CTG 1392
Ile His Trp Tyr Ala Asn Ala Leu Tyr Lys Val Lys Asp Tyr Val Leu
450 455 460

35 CCG CAG AGC TGG CGC CAC GAT TGA 1416
Pro Gln Ser Trp Arg His Asp
465 470

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 471 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

45 Met Asp Asn His Ser Ser Val Pro Trp Ala Ser Ala Ala Ser Val Thr
1 5 10 15

Cys Leu Ser Leu Gly Cys Gln Met Pro Gln Phe Gln Phe Gln Phe Gln
20 25 30

50 Leu Gln Ile Arg Ser Glu Leu His Leu Arg Lys Pro Ala Arg Arg Thr
35 40 45

55 Gln Thr Met Arg His Ile Ala His Thr Gln Arg Cys Leu Ser Arg Leu
50 55 60

Thr Ser Leu Val Ala Leu Leu Leu Ile Val Leu Pro Met Val Phe Ser
65 70 75 80

60 Pro Ala His Ser Cys Gly Pro Gly Arg Gly Leu Gly Arg His Arg Ala

- 96 -

405 410 415

Met Arg Leu Leu Ser Thr Leu Glu Ala Trp Leu Pro Ala Lys Glu Gln
420 425 430

5 Leu His Ser Ser Pro Lys Val Val Ser Ser Ala Gln Gln Gln Asn Gly
435 440 445

10 Ile His Trp Tyr Ala Asn Ala Leu Tyr Lys Val Lys Asp Tyr Val Leu
450 455 460

Pro Gln Ser Trp Arg His Asp
465 470

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 221 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Cys Gly Pro Gly Arg Gly Xaa Gly Xaa Arg Arg His Pro Lys Lys Leu
1 5 10 15

Thr Pro Leu Ala Tyr Lys Gln Phe Ile Pro Asn Val Ala Glu Lys Thr
20 25 30

Leu Gly Ala Ser Gly Arg Tyr Glu Gly Lys Ile Xaa Arg Asn Ser Glu
35 40 45

Arg Phe Lys Glu Leu Thr Pro Asn Tyr Asn Pro Asp Ile Ile Phe Lys
50 55 60

Asp Glu Glu Asn Thr Gly Ala Asp Arg Leu Met Thr Gln Arg Cys Lys
65 70 75 80

Asp Lys Leu Asn Xaa Leu Ala Ile Ser Val Met Asn Xaa Trp Pro Gly
85 90 95

Val Xaa Leu Arg Val Thr Glu Gly Trp Asp Glu Asp Gly His His Xaa
100 105 110

Glu Glu Ser Leu His Tyr Glu Gly Arg Ala Val Asp Ile Thr Thr Ser
115 120 125

Asp Arg Asp Xaa Ser Lys Tyr Gly Xaa Leu Xaa Arg Leu Ala Val Glu
130 135 140

Ala Gly Phe Asp Trp Val Tyr Tyr Glu Ser Lys Ala His Ile His Cys
145 150 155 160

Ser Val Lys Ala Glu Asn Ser Val Ala Ala Lys Ser Gly Gly Cys Phe

				165					170					175
	Pro	Gly	Ser	Ala	Xaa	Val	Xaa	Leu	Xaa	Xaa	Gly	Gly	Xaa	Lys
				180					185					190
5	Lys	Asp	Leu	Xaa	Pro	Gly	Asp	Xaa	Val	Leu	Ala	Ala	Asp	Xaa
			195					200					205	Xaa
10	Xaa	Leu	Xaa	Xaa	Ser	Asp	Phe	Xaa	Xaa	Phe	Xaa	Asp	Arg	
		210					215					220		

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 167 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Cys	Gly	Pro	Gly	Arg	Gly	Xaa	Xaa	Xaa	Arg	Arg	Xaa	Xaa	Xaa	Pro	Lys
1				5					10					15	
Xaa	Leu	Xaa	Pro	Leu	Xaa	Tyr	Lys	Gln	Phe	Xaa	Pro	Xaa	Xaa	Xaa	Glu
			20					25					30		
Xaa	Thr	Leu	Gly	Ala	Ser	Gly	Xaa	Xaa	Glu	Gly	Xaa	Xaa	Xaa	Arg	Xaa
		35					40					45			
Ser	Glu	Arg	Phe	Xaa	Xaa	Leu	Thr	Pro	Asn	Tyr	Asn	Pro	Asp	Ile	Ile
	50					55					60				
Phe	Lys	Asp	Glu	Glu	Asn	Xaa	Gly	Ala	Asp	Arg	Leu	Met	Thr	Xaa	Arg
	65				70				75						80
Cys	Lys	Xaa	Xaa	Xaa	Asn	Xaa	Leu	Ala	Ile	Ser	Val	Met	Asn	Xaa	Trp
				85					90					95	
Pro	Gly	Val	Xaa	Leu	Arg	Val	Thr	Glu	Gly	Xaa	Asp	Glu	Asp	Gly	His
			100					105					110		
His	Xaa	Xaa	Xaa	Ser	Leu	His	Tyr	Glu	Gly	Arg	Ala	Xaa	Asp	Ile	Thr
			115				120						125		
Thr	Ser	Asp	Arg	Asp	Xaa	Xaa	Lys	Tyr	Gly	Xaa	Leu	Xaa	Arg	Leu	Ala
		130				135					140				
Val	Glu	Ala	Gly	Phe	Asp	Trp	Val	Tyr	Tyr	Glu	Ser	Xaa	Xaa	His	Xaa
					150					155					160
His	Xaa	Ser	Val	Lys	Xaa	Xaa									
				165											